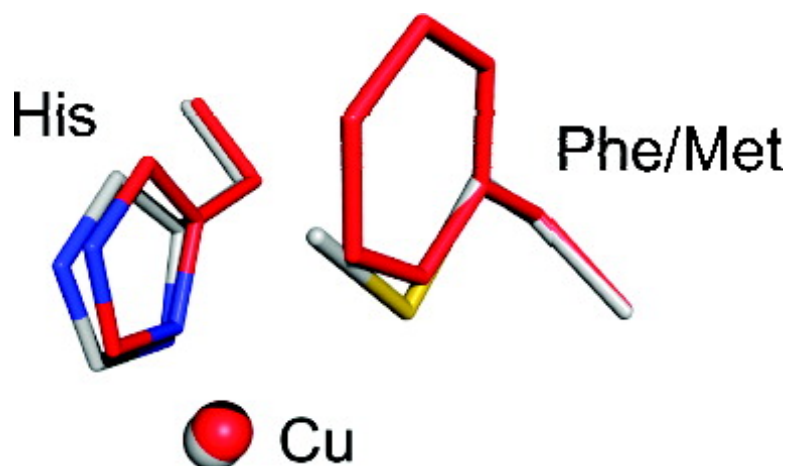Article

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π-Interaction Tuning of the Active Site Properties of Metalloproteins

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Abstract: The influence of π-interactions with a His ligand have been investigated in a family of copper-containing redox metalloproteins. The Met16Phe and Met16Trp pseudoazurin, and Leu12Phe spinach and Leu14Phe *Phormidium laminosum* plastocyanin variants possess active-site π-contacts between the introduced residue and His81 and His87/92 respectively. The striking overlap of the side chain of Phe16 in the Met16Phe variant and that of Met16 in wild type pseudoazurin identifies that this position provides an important second coordination sphere interaction in both cases. His-ligand protonation and dissociation from Cu(I) occurs in the wild type proteins resulting in diminished redox activity, providing a [H$^+$]-driven switch for regulating electron transfer. The introduced π-interaction has opposing effects on the pK_a for the His ligand in pseudoazurin and plastocyanin due to subtle differences in the π-contact, stabilizing the coordinated form of pseudoazurin whereas in plastocyanin protonation and dissociation is favored. Replacement of Pro36, a residue that has been suggested to facilitate structural changes upon His ligand protonation, with a Gly, has little effect on the pK_a of His87 in spinach plastocyanin. The mutations at Met16 have a significant influence on the reduction potential of pseudoazurin. Electron self-exchange is enhanced, whereas association with the physiological partner, nitrite reductase, is only affected by the Met16Phe mutation, but k_{cat} is halved in both the Met16Phe and Met16Trp variants. Protonation of the His ligand is the feature most affected by the introduction of a π-interaction.

Introduction

The amino acids that bind the cofactor in a metalloprotein are usually essential for structure and function. A large number of mutagenesis studies have focused on these ligating residues and most have found that alterations in the first coordination sphere have a drastic effect on reactivity. The intricate nature of a folded protein results in the active sites of metalloproteins possessing many additional interactions outside the immediate ligand environment. In catalytic metalloproteins the importance of this second coordination sphere (roughly within a 6 Å radius of the metal) for tuning substrate specificity and participating in substrate turnover is well appreciated.[1,2] However, the role of the second coordination sphere in other types of metal sites in proteins, for example those involved in conceptually more simple reactions such as electron and metal transfer, is less obvious. As more studies are undertaken some underlying principles are beginning to emerge. For example, the reduction potential (E_m) of electron transfer (ET) proteins,[3–15] and metal

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transfer in metallochaperones,[16,17] are both influenced by hydrogen bonding to the ligating amino acids, mainly the thiolate sulfur of Cys residues. The structural importance of π-interactions in biological and chemical systems is well appreciated[18−21] and their involvement in the second coordination sphere of a number of metalloproteins has been noted.[19,22−25] A short Cu(I) to Trp distance has recently been identified in CusF, a protein involved in copper homeostasis, which has been described as a cation-π interaction.[26]

In this study we use ET metalloproteins to assess the importance of π-interactions provided by residues in the second coordination sphere. The system we have chosen is the well-studied family of small copper-containing redox shuttles (the cupredoxins), which possess a mononuclear type 1 (T1) copper site anchored to a stable β-barrel scaffold.[27−30] Two His residues and a Cys strongly bind the metal with coordination usually (and in all of the proteins included in this work) completed by a weak axially interacting Met. Plastocyanin (PC), the cupredoxin involved in photosynthetic ET, from the fern *Dryopteris crassirhizoma* possesses a π-interaction between a phenyl group and the coordinating His90.[24,25] It was suggested that this contact could influence active-site properties, and in particular protonation of the C-terminal His ligand, a feature known to occur in certain Cu(I) cupredoxins, including PC.[24,31] The protonated form has diminished redox activity[32,33] and this effect therefore provides a switch via which photosynthetic ET (and other ET chains involving cupredoxins) could be controlled by [H⁺].[32−34] A recent study on *D. crassirhizoma* PC has found that the His90 ligand protonates with a pK_a lower than that typically

observed for a PC and at a pH at which the protein is not stable.[35] Surprisingly, mutation of Phe12, which forms the π-interaction with His90, to a Leu (the residue usually found in this position in PCs) has no effect on the pK_a value.[35] The role of π-interactions between a phenyl group and one of the ligating His residues has been investigated in other cupredoxins. In the case of pseudoazurin (PAZ), which is involved in ET in denitrifying bacteria, the introduction of a Phe in place of Met16 adjacent to the coordinating His81 results in a decrease in the pK_a for this ligand.[36]

The surface surrounding the protonatable His ligand is hydrophobic and is thought to be the main surface via which cupredoxins interact with partners,[37−42] with the His implicated in the ET pathway. The presence of a π-interaction with this ligand will influence the hydrophobic patch and could impact on ET. To investigate the importance of π-interactions involving ligating residues in ET metalloproteins, and to resolve the importance of such a contact for His ligand protonation in cupredoxins, mutagenesis studies of PC and PAZ have been undertaken. π-interactions, verified crystallographically, influence E_m values, ET reactivity with partners, and most significantly, the pK_a of the C-terminal His ligand.

Materials and Methods

Protein Samples. *Escherichia coli* strain JM105 was transformed with either pTrcPAZ [pTrc99A derivative harboring the gene for wild type (WT) *Achromobacter cycloclastes* PAZ including the transit peptide], pTrcM16F_PAZ[36] or pTrcM16W_PAZ[43] (pTrc99A derivatives harboring the Met16Phe and Met16Trp PAZ genes respectively also including the transit peptide), and cells were grown, harvested and proteins isolated and purified as described previously.[36,43] The copper containing nitrite reductase (NIR) from *A. cycloclastes*[44] and the Leu14Phe *Phormidium laminosum* PC variant[45] were prepared as described previously. pTrc99a derivatives harboring the genes for WT and Leu12Phe spinach PC were provided by Dr. Klaus Bernauer (Université de Neuchâtel, Switzerland), while that for the Pro36Gly variant cloned in a similar manner, but in a pUC18 derivative,[46] was obtained from Dr. Örjan Hansson (Göteborg University, Sweden). Proteins were overexpressed in JM105 (Leu12Phe) and TG1 (WT and Pro36Gly) *E. coli* and purified as described previously.[47]

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Table 1. Properties of the Proteins Studied

parameter	WT PAZ[a]	Met16Phe PAZ[a]	Met16Trp PAZ	WT spinach PC[b]	Leu12Phe spinach PC	Pro36Gly spinach PC
UV/vis						
λ_1 [ε_1 ($M^{-1}cm^{-1}$)]	594 [3100]	598 [3500]	595 [3600]	597	597	597
λ_2 [ε_2 ($M^{-1}cm^{-1}$)]	454 [1330]	459 [1050]	458 [1140]	~460	~460	~460
$\varepsilon_2/\varepsilon_1$	0.43	0.30	0.32			
EPR[c]	rh	rh	rh			
E_m (mV)[d]	263	319	305	378	379	398
pK_a (NMR)[e]	~4.5	~4.1	~4.2	4.9 ± 0.1	5.6 ± 0.1	5.1 ± 0.1
pK_a (CV)[e]					5.7 ± 0.1	5.1 ± 0.1
k_{ESE} ($M^{-1}s^{-1}$)[f]	3.1×10^3	7.3×10^3	9.9×10^3			
reaction with NIR[g]						
K_M (μM)	13 ± 2	26 ± 3	17 ± 3			
k_{cat} (s^{-1})	113 ± 3	57 ± 3	53 ± 3			

[a] Taken from ref 36. [b] Taken from ref 47. [c] rh = rhombic, ax = axial. EPR parameters derived from simulations using the program SimFonia (Bruker) are; WT PAZ: $g_x = 2.015$, $g_y = 2.053$, $g_z = 2.213$, $A_x = 7.3$ mT, $A_y = 1.8$ mT, $A_z = 3.5$ mT; Met16Phe PAZ: $g_x = 2.015$, $g_y = 2.043$, $g_z = 2.206$, $A_x = 7.5$ mT, $A_z = 3.8$ mT; Met16Trp PAZ: $g_x = 2.013$, $g_y = 2.047$, $g_z = 2.210$, $A_x = 7.5$ mT, $A_y = 1.0$ mT, $A_z = 4.0$ mT. [d] Measured at pH 8. [e] For His81 in PAZ and His87 in PC measured by NMR spectroscopy and cyclic voltammetry (CV). pK_a (NMR) values of 5.1 and 5.6 respectively are obtained for His92 in WT and Leu14Phe *P. laminosum* PC. [f] ESE rate constants measured at pH* 7.6 and 25 °C. [g] Data from the analysis of steady-state kinetics of the oxidation of Cu(I) WT, Met16Phe and Met16Trp PAZ by NIR at pH 7 and 25 °C.

UV/Vis Spectrophotometry. UV/vis spectra were acquired at 25 °C on a Perkin-Elmer λ35 or a Shimadzu UV-2101PC spectrophotometer with the proteins typically in 10 mM phosphate pH 8.0.

Metal Concentration Determinations and Molecular Weights. The concentration of copper and zinc in samples were measured as described previously and molecular weights were determined by mass spectrometry (see Supporting Information).[48]

Spectroscopy. X-band EPR and ^1H NMR (at 500.13 MHz) spectra were obtained using Cu(II) (EPR and NMR) and Cu(I) proteins (^1H NMR only) as reported previously (see Supporting Information).[47,49] ^1H NMR spectra of Met16Trp PAZ at various pH values were obtained both in 10 mM phosphate at 25 °C and also in 100 mM phosphate at 40 °C. Samples of Met16Trp PAZ for electron self-exchange (ESE, the reaction whereby the two oxidation states of a redox couple interconvert[30]), rate constant (k_{ESE}) measurements were made as described previously[49] with the concentration of Cu(II) protein determined using the ε values shown in Table 1.

Electrochemical Measurements. E_m values were measured at ambient temperature (22 ± 1 °C) using cyclic voltammetry (see Supporting Information).[31] All E_m values are referenced to the NHE, and voltammograms were calibrated using the [Co(phen)$_3$]$^{3+/2+}$ couple [370 mV vs NHE].

Oxidation of Cu(I) WT, Met16Phe and Met16Trp PAZ by NIR. WT, Met16Phe and Met16Trp PAZ in 20 mM phosphate pH 7.0 were reduced by adding one equivalent of ascorbate with reductant removed on a HiTrap desalting column (GE Healthcare). An aliquot of a KNO$_2$ solution was added to give a final concentration of 2 mM (protein concentrations ~ 20−200 μM) in 20 mM phosphate pH 7.0. NIR (20 μM) was incubated with 200 μM CuSO$_4$ in 10 mM Tris pH 8.0 for one week to ensure full occupancy of copper at the type 2 (T2) site. The excess copper was removed by ultrafiltration and the NIR solution exchanged into 20 mM phosphate pH 7.0. All solutions used for kinetic studies were degassed and kept under nitrogen.

The reaction between PAZ and NIR was initiated by adding NIR (<0.5 nM) into a reaction solution which contained PAZ (~20−200 μM) and 2 mM KNO$_2$ in degassed 20 mM phosphate pH 7.0. The oxidation of Cu(I) WT, Met16Phe and Met16Trp PAZ was monitored (25 °C) at 594, 598 and 595 nm, respectively. Control experiments excluding NIR were carried out to check for air

oxidation of Cu(I) PAZ, and all measurements were performed in triplicate. The concentration of NIR was determined using $\varepsilon_{460} = 2400$ $M^{-1}cm^{-1}$,[44] while WT PAZ and variants were quantified using the ε values listed in Table 1. The concentration of Cu(I) PAZ in the reaction was determined by fully oxidizing the mixture at the end of the experiment with an excess of K$_3$[Fe(CN)$_6$] and subtracting the concentration of Cu(II) PAZ just before the addition of NIR [oxidized NIR has an absorption maximum at 590 nm (ε_{590} = 1800 $M^{-1}cm^{-1}$) but its concentration is too low (<0.5 nM) to interfere with this determination].

Protein Crystallization and X-Ray Data Collection. Crystals of Cu(II) Met16Phe PAZ were obtained by the hanging drop method of vapor diffusion using 1.5 μL of protein in 5 mM Tris pH 7.5 mixed with 1.5 μL of 35% PEG 4000. Crystals were frozen directly in a nitrogen stream with no additional cryo-protection. Diffraction data for Met16Phe PAZ were collected at 100 K at the Daresbury-SRS (station 10.1) on a mar225 CCD detector. Data were processed with MOSFLM[50] and scaled with SCALA.[51] Five percent of data were set aside for calculation of R_{free}, and data collection and processing statistics are given in Table 2.

Crystals of Leu14Phe PC were obtained as described previously[45] from the following conditions; (A) 10% PEG 8000, (B) 15% PEG 8000 plus 0.2 M MgCl$_2$ and (C) 10% PEG 8000 with 0.2 M zinc acetate (A at 32 °C, B and C at 21 °C). In all cases the buffer was 0.1 M sodium cacodylate pH 6.5. The crystals obtained in (A) were colorless indicating that reduction of the copper occurred during crystal growth. The crystals in (B) and (C) remained oxidized (i.e., blue). Prior to data collection a Cu(II) crystal from (C) was reduced by transferring it to a new drop containing the same precipitant but at lower pH (0.1 M sodium cacodylate pH 5.1) supplemented with sodium ascorbate (0.1 mM). Reduction was considered to be complete when the crystal became completely colorless (<5 min). Cryo-protection prior to freezing Leu14Phe crystals and data collection are described elsewhere.[45]

Structure Solution and Refinement. The structure of Met16Phe PAZ was solved by molecular replacement using MOLREP (as implemented in CCP4[52]) with the structure of WT PAZ (PDB entry 1bqk[53]) as the search model. Iterative model building (using COOT[54])

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Table 2. Crystallographic Data Collection, Processing and Refinement Statistics for Met16Phe PAZ

	Cu(II) Met16Phe PAZ
Data Collection[a]	
instrumentation	Daresbury-SRS, station 10.1
wavelength (Å)	1.283
space group	$P2_1$
resolution range (Å)	25.50−1.60 (1.69−1.60)
unit cell parameters (Å)	$a = 34.93$, $b = 90.54$, $c = 35.22$, $\beta = 98.55°$
no. of unique reflections	28533 (4142)
redundancy	4.9 (4.8)
$I/\sigma(I)$	16.7 (4.9)
completeness (%)	100.0 (100.0)
R_{merge} (%)	6.4 (27.2)
Refinement[a]	
resolution	25.50−1.60 (1.64−1.60)
R_{factor} (%)	13.1 (15.8)
R_{free} (%)	18.0 (26.9)
rmsd bond lengths (Å)	0.014
rmsd bond angles (deg)	1.45
no. of non-hydrogen atoms	2295
average B-factor (protein − Å^2)	12.8
average B-factor (ligands − Å^2)	26.6
Ramachandran favored (%)	98.8

[a] Values in parentheses represent data for the highest resolution shell.

and refinement [(REFMAC5[55]) as implemented in CCP4] cycles were used to complete the structure. Refinement statistics are given in Table 2. Hydrogen atoms were added at riding positions and anisotropic B-factors were refined while monitoring both the R_{factor} and R_{free}. Ramachandran analysis used MOLPROBITY.[56] Structure solution and refinement for the three different forms of Leu14Phe PC has been described previously.[45] Structures were obtained at resolutions of 1.72, 1.40 and 1.50 Å respectively for the crystals obtained from conditions A, B and C. LSQMAN[57] was used to generate superimposed structures and determine root-mean-square deviations (rmsds) for C$^\alpha$ atoms. For the overlays including *D. crassirhizoma* PC and for that between Met16Phe PAZ and Leu14Phe spinach PC the Secondary Structure Matching algorithm in COOT was used. Figures of protein structures were prepared with Pymol (http://www.pymol.org). Crystallographic coordinates have been deposited in the Protein Data Bank with PDB ID codes 2jkw [Cu(II) Met16Phe PAZ], 3cvb [Cu(I) Leu14Phe PC, pH 6.5], 3cvc [Cu(II) Leu14Phe PC], and 3cvd [Cu(I) Leu14Phe PC, pH 5.1].

Results

The Met16Phe and Met16Trp PAZ, and the Leu12/14Phe spinach/*P. laminosum* PC variants were all constructed with the aim of introducing a π-interaction with the C-terminal His ligand (an interaction that is present between Phe12 and the His90 ligand in *D. crassirhizoma* PC). Pro36 is conserved in almost all PCs, and has been identified as playing a potentially important role in His ligand protonation,[32] but is a Gly in the *D. crassirhizoma* protein.[24] We have therefore also analyzed the influence of the Pro36Gly mutation in spinach PC.

Structure of Cu(II) Met16Phe PAZ. The overall structure of Met16Phe PAZ contains two molecules in the asymmetric unit which superimpose with an rmsd of 0.56 Å for the C$^\alpha$ atoms.

Four crystal packing interfaces are present which bury ≥ 250 Å^2 surface per protein chain. There are no crystal contacts in the vicinity of His81 and the nearest symmetry related atom is > 7 Å away. The structure of Met16Phe PAZ (1.60 Å resolution) exhibits a high degree of similarity to the 1.35 Å structure of the WT protein (1bqk)[53] with rmsds of 0.37 and 0.56 Å for chains A and B respectively. However, the crystal packing arrangement for WT PAZ (crystallized from ∼70% saturated ammonium sulfate) is significantly different. The asymmetric unit is composed of a single protein chain and there are only two crystal packing interfaces that bury ≥ 250 Å^2. One of these interfaces involves the hydrophobic patch and places His81 ∼4 Å from Pro71 of the neighboring monomer.

The introduction of a Phe in place of Met16 adjacent to the His81 ligand results in minor structural alterations around the metal site of PAZ. The most significant change is that the methyl group of the surface exposed Met84 moves away from the bulky phenyl moiety (Met84 is modeled in two conformations in the A and B chains in the Met16Phe PAZ structure, both associated with movement of its side chain away from Phe16). The phenyl ring of the introduced Phe16 is parallel to the imidazole of His81 indicating a significant π-interaction, with the planes of the two rings approximately 3.7−4.2 Å apart (measured from the His81 C$^{\delta 2}$ atom to the C atoms of the phenyl ring in chains A and B) (see Figure 1). This arrangement closely matches that in *D. crassirhizoma* PC[24] where the His90 C$^{\delta 2}$ is 3.4 to 3.8 Å from the phenyl ring C atoms of Phe12 (see Figure 1). The superposition of WT and Met16Phe PAZ structures reveals a striking overlap between the Met and Phe side chains (Figure 1). The active site geometry and the Cu(II) to ligand bond lengths are almost unaltered by the Met16Phe mutation in PAZ (see Table S1, Supporting Information).

Active Site Structure of Cu(II) Leu14Phe *P. laminosum* PC. In the Cu(II) structure of Leu14Phe *P. laminosum* PC the asymmetric unit consists of a single chain. The overall structure is similar to that of the WT protein[58] (chain A) with an rmsd of 0.63 Å for the C$^\alpha$ overlay. Structural differences, which most likely occur from crystal packing effects, are found at loop 1 on which the mutated residue is located, and also in the recognized irregular region of PC (residues 44−60).[45] The phenyl ring of Phe14 lies parallel to the imidazole of the His92 ligand, again in an arrangement reminiscent of that seen in the *D. crassirhizoma* protein (see Figure 2). A ring-to-ring separation of 3.6−3.9 Å (measured from the His92 C$^{\delta 2}$ atom to the C atoms of the phenyl ring) is found for Leu14Phe PC. The introduction of this π-interaction also has a limited effect on the Cu(II) site geometry of *P. laminosum* PC (see Table S2, Supporting Information).

Active Site Structure of Cu(I) Leu14Phe *P. laminosum* PC. Two structures of Cu(I) Leu14Phe *P. laminosum* PC have been determined, one from a crystal which had reduced during crystallization (at pH 6.5 and 32 °C) while the other was obtained from a crystal grown at 21 °C and pH 6.5 in the presence of 200 mM Zn(II) and then reduced at pH 5.1. These conditions give rise to two different crystal forms of the variant, with the former having two molecules in the asymmetric unit while the latter has three chains present [similar to the structure of the Cu(II) WT protein which was also crystallized in the presence of Zn(II)[58]]. The packing arrangements in the different crystal forms of Leu14Phe

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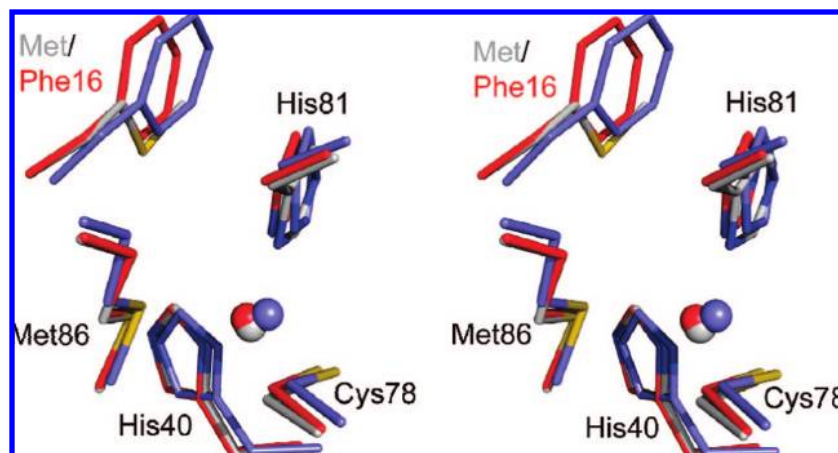
Figure 1. Stereoview showing an overlay of the Cu(II) site structures of WT PAZ (gray), Met16Phe PAZ (chain A, red) and *D. crassirhizoma* PC (slate). In PAZ the thioether side-chain of Met16 packs against the His81 ligand.
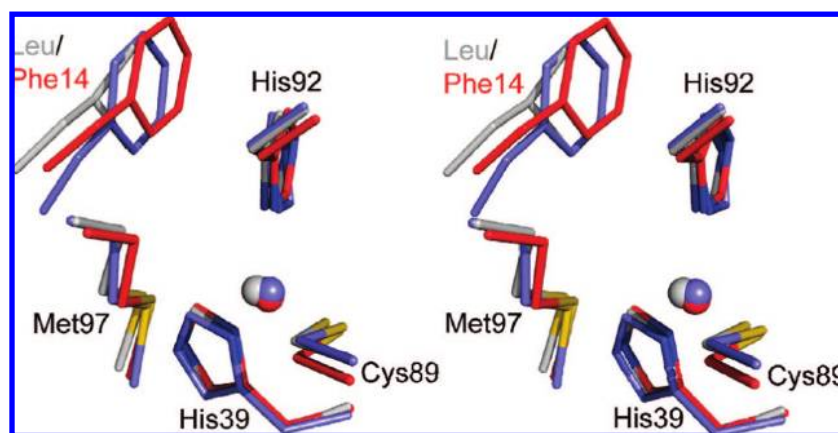

Figure 2. Stereoview showing an overlay of the Cu(II) site structures of WT *P. laminosum* PC (chain A, gray), the Leu14Phe variant (red) and *D. crassirhizoma* PC (slate). In *P. laminosum* PC the side-chain of Leu14 packs against the His92 ligand.

PC have been discussed elsewhere.[45] It is important to note here that none of the interfaces in the different crystal forms involve either Phe14 or the His92 ligand. The overall structure of Cu(I) Leu14Phe PC is similar in the two crystal forms (rmsd of 0.67 Å for a C^{α} overlay of the A chains) and to that of the Cu(II) protein [rmsds of 0.42 and 0.68 Å for C^{α} overlays of the Cu(II) protein with the A chains of the Cu(I) structures at pH 6.5 and 5.1, respectively].[45] Differences are found between the Cu(II) and all of the Cu(I) sites in both the pH 6.5 and 5.1 structures. The electron density at the Cu(I) sites is best fit by modeling two orientations of the His92 ligand; one binding Cu(I), and a second with the imidazole ring rotated by 180° (see Figure 3). The active site geometry of the conformer with His92 coordinated is similar to that of the Cu(II) protein (see Table S2, Supporting Information). The Cu–$N^{\delta 1}$(His92) bond has lengthened by ∼0.2 Å while the copper ion has moved further from the N_2S plane toward Met97 [the Cu–S(Met97) bond decreases by ∼0.3 Å], and away from Pro38. The second conformer positions the $N^{\delta 1}$ atom of His92 ∼5 Å from the copper with the $C^{\delta 2}$ atom pointing toward the metal (∼3 Å away) (see Figure 3). The presence of two conformations is supported, in part, by the arrangement of water molecules around the active site although this cannot be taken as a definitive indicator of orientation in these structures. These observations are all consistent with the onset of protonation of the His92 ligand, which has been analyzed crystallographically for poplar PC[32] and other

cupredoxins, including PAZ.[48,59,60] There is further evidence of His92 protonation in the pH 5.1 structure as in chains A and C the electron density for the copper ion is somewhat ellipsoidal (not distinct peaks) and two positions could potentially be modeled. This feature has been observed in other cupredoxin structures where the equivalent protonated His ligand adopts two conformations.[12,48] However, in Leu14Phe PC the electron density does not conclusively support two distinct positions for the copper ion.

Spectroscopic Properties. All of the mutations made have a limited effect on the spectroscopic properties measured for the Cu(II) proteins (see Table 1 and Figures S1–S3 in the Supporting Information). Copper concentrations from AAS data were used to determine ε values of the main S(Cys)→Cu(II) LMCT bands for the proteins used in kinetic studies (WT, Met16Phe and Met16Trp PAZ). The ^{1}H NMR spectra of the Cu(I) variants are similar to those of the WT proteins. However, the mutations do influence the chemical shift values of the C-terminal His ligand, with the $C^{\delta 2}$H resonance exhibiting the largest changes (see Supporting Information). These can be ascribed to ring current effects and provide additional evidence for a significant π-interaction between the introduced aromatic residues and the His ligand.

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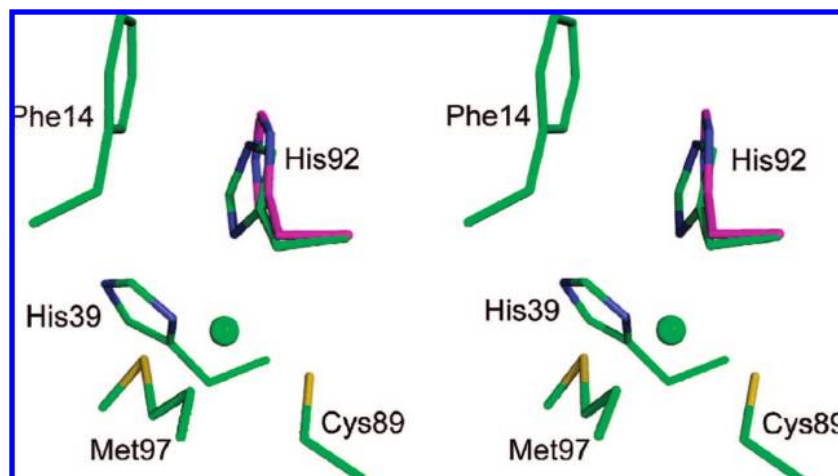
Figure 3. Stereoview showing the Cu(I) site structure of Leu14Phe *P. laminosum* PC (chain A of the pH 5.1 structure) with the second ("rotated") conformation of the His92 ligand included in magenta. The angle between the Phe14 phenyl group and the imidazole ring is 27° when His92 is coordinated compared to 15° in the protonated conformer.

His Ligand Protonation Monitored by ^{1}H NMR Spectroscopy. The effect of pH on the ^{1}H NMR spectra of all Cu(I) proteins has been investigated. In particular, the influence of pH on the His ligand resonances, and signals from amino acids close to the active site, can be used to monitor the protonation state of the C-terminal His ligand in cupredoxins (and to determine the pK_a value).[35,39,47,61−66] In the NMR spectra of Cu(I) WT, Met16Phe and Met16Trp PAZ, the His ligand resonances (particularly those of His81) broaden as the pH is lowered making detection difficult at more acidic values (this is thought to be due to either an intermediate rate of exchange between the protonated and deprotonated forms of His81, or the presence of two forms of the protonated His that are exchanging at an intermediate rate on the NMR time scale).[36,64] The His40 $C^{\delta 2}$H resonance can be observed in the pH* (pH measured in D$_2$O uncorrected for the deuterium isotope effect) range 6.7 to 3.6 and a fit (see eq 1, where δ_H and δ_L are the chemical shift values at high and low pH respectively and K_a is the acid dissociation constant) of the pH* dependence of its chemical shift gives a pK_a* of 4.5 ± 0.1 (see Figure S4, Supporting Information). This is consistent with a pK_a of 4.9 ± 0.1 for His81 obtained from kinetic studies[36] and 4.8 ± 0.1 from NMR investigations on *Alcaligenes faecalis* PAZ.[63] The incomplete pH* profiles for the His81 imidazole ring resonances in WT PAZ fit well using a pK_a* of 4.5 (see Figure S5, Supporting Information). NMR data for the $C^{\varepsilon 1}$H and $C^{\delta 2}$H resonances of His81 in Met16Phe PAZ[36] can be fit (see Figure S6, Supporting Information) to give pK_a* values of 4.1 ± 0.1 and 4.0 ± 0.1 respectively (consistent with a 0.4 pH unit decrease in the pK_a of His81 in Met16Phe PAZ determined from kinetic studies[36]). Similar fits (see Figure S7, Supporting Information) for the His81 signals of the Met16Trp PAZ variant gives pK_a*s of 4.2 ± 0.1 and 4.3 ± 0.1 respectively. In both cases fitting requires the chemical shift at low pH to be fixed and the values obtained for the WT protein have been used. The chemical shift of the His81
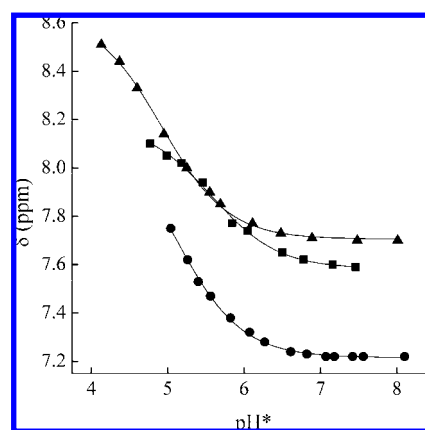
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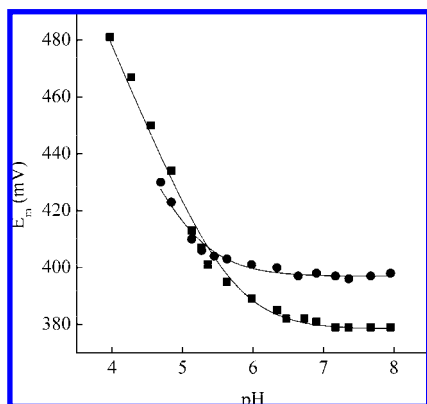(66) Hass, M. A. S.; Christensen, H. E. M.; Zhang, J.; Led, J. J. *Biochemistry* **2007**, *46*, 14619−14628.

Figure 4. Dependence (25 °C) on pH* of the chemical shift of the His87 $C^{\varepsilon 1}$H resonance in the NMR spectra of WT (▲), Leu12Phe (■) and Pro36Gly (●) spinach PC. The solid lines are fits of the data to eq 1 which yield pK_a* values of 4.9 ± 0.1, 5.6 ± 0.1 and 5.1 ± 0.1, respectively.

$C^{\delta 2}$H proton resonance is influenced by the mutations made which could cause an additional error in the fits. Broadening of the His ligand resonances was less severe for Met16Trp PAZ in 100 mM phosphate at 40 °C allowing pK_a*s of 4.1 ± 0.1 and 4.0 ± 0.3 for the pH* dependence of the chemical shift values of the His81 $C^{\delta 2}$H and $C^{\varepsilon 1}$H resonances respectively (see Figure S8, Supporting Information) to be determined (chemical shift values at low pH were not fixed in these fits). A similar fit for the Met16Phe His81 $C^{\varepsilon 1}$H data (10 mM phosphate and 25 °C) gives a pK_a* of 4.3 ± 0.1 (data not shown). These data demonstrate that the Met16Phe and Met16Trp mutations result in a lower pK_a for His81 in Cu(I) PAZ.

$$\delta = (K_a\delta_H + [H^+]\delta_L)/(K_a + [H^+]) \qquad (1)$$

The pH* dependence of the chemical shift of the resolved His87 $C^{\varepsilon 1}$H resonance in WT[47] and Leu12Phe spinach PC results in pK_a*s of 4.9 ± 0.1 and 5.6 ± 0.1 respectively (Figure 4). For *P. laminosum* PC the pH dependence of the His39 $N^{\varepsilon 2}$H resonance gives a pK_a of 5.1 ± 0.1 for His92 in the WT protein, consistent with that obtained from the pH dependence of a range of amide ^{15}N resonances (5.1 ± 0.1),[39] and 5.6 ± 0.1 for the Leu14Phe variant (see Figure S9, Supporting Information). In the Pro36Gly spinach PC variant

Figure 5. Dependence on pH ($I = 0.10$ M, NaCl) of the reduction potential (E_m) of Leu12Phe (■) and Pro36Gly (●) spinach PC. All values are referenced to the NHE at 22 °C, and the solid lines are fits of the data to eq 2 which yield pK_a^{red} values of 5.7 ± 0.1 and 5.1 ± 0.1, respectively.

the pH* dependence of the His87 $C^{\varepsilon 1}H$ resonance results in a pK_a^* of 5.1 ± 0.1 (see Figure 4).

Influence of π-Interactions on Reduction Potentials and their pH Dependence. We have previously shown that the Met16Phe mutation results in the E_m of PAZ increasing from 263 mV to 319 mV at pH ~ 8.[36] The Met16Trp mutation causes a smaller increase to 305 mV at pH 8. The E_m of Leu12Phe spinach PC is 379 mV, compared to 378 mV for the WT protein, at pH 8 (a limited effect has been found for the Leu14Phe *P. laminosum* PC mutation). The Pro36Gly mutation has a larger effect on E_m with a value of 398 mV obtained at pH 8. The pH dependence of E_m has been studied previously to analyze His ligand protonation in spinach PC.[67–69] We have therefore also used this approach to determine the pK_a for His87 in the Leu12Phe and Pro36Gly variants. The data obtained are shown in Figure 5 and were fit to eq 2;

$$E_m(pH) = E_m(\text{high pH}) + (RT/nF)\ln(1 + [H^+]/K_{red}^a) \quad (2)$$

where $E_m(pH)$ is the measured E_m, $E_m(\text{high pH})$ is the E_m at high pH, K_a^{red} is the dissociation constant for the residue in the reduced protein which affects E_m as the pH is lowered and the other symbols have their usual meaning. These fits provide pK_a^{red} values of 5.7 ± 0.1 and 5.1 ± 0.1 for Leu12Phe and Pro36Gly PC respectively, which are in excellent agreement with the NMR data (Figure 4).

Effect of π-Interactions on ET Reactivity Measured using the Electron Self Exchange Reaction. Corrected k_{ESE} values for WT and Met16Phe PAZ of 3.1×10^3 M^{-1}s^{-1} and 7.3×10^3 M^{-1}s^{-1} at pH* 7.6 ($I = 0.10$ M) are reported (see Supporting Information). For Met16Trp PAZ k_1 values (from slopes of the influence of [Cu(II)] on T_1^{-1}) of 9.6×10^3 M^{-1}s^{-1}, 1.0×10^4 M^{-1}s^{-1} and 1.0×10^4 M^{-1}s^{-1} are obtained from the His81 $C^{\delta 2}H$, His81 $C^{\varepsilon 1}H$ and His40 $C^{\varepsilon 1}H$ proton resonances respectively (see Figure S10, Supporting Information). These yield a k_{ESE} (average at 25 °C) of 9.9×10^3 M^{-1}s^{-1} at pH* 7.6 ($I = 0.10$ M). The introduction of a π-interaction at the active site of PAZ enhances ESE reactivity with the magnitude of this effect largest when a Trp is introduced adjacent to the His81 ligand.

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Influence of Active-Site π-Interactions on the ET Reaction Between PAZ and NIR. The copper-containing NIR from *A. cycloclastes* possesses a T2 copper site at which nitrite is reduced and a T1 copper center that accepts electrons from PAZ. The steady-state kinetics of the oxidation of Cu(I) WT, Met16Phe and Met16Trp PAZ by NIR have been investigated. Analysis of Lineweaver−Burk plots (see Figures S11−S13, Supporting Information) yield K_m values of 13 ± 2, 26 ± 3 and 17 ± 3 μM respectively. The V_{max} values obtained from these plots give rise to k_{cat} values of 113 ± 14, 57 ± 9 and 53 ± 8 s^{-1} for WT, Met16Phe and Met16Trp PAZ respectively (K_m and k_{cat} for the WT protein correspond favorably with values reported previously[70]). The introduction of a Phe at position 16 in PAZ disfavors complex formation with NIR, while the change to Trp has very little effect. k_{cat} values indicate that ET reactivity is decreased in both variants compared to the WT protein.

Discussion

The importance of second coordination sphere residues for metal sites in proteins has been assessed using a family of ET proteins for which detailed structural data is available. One aspect of this study has been the influence of π-interactions on His ligand protonation and dissociation in Cu(I) PAZ and PC. The production of a form of the protein with diminished redox activity upon protonation means that this process could provide a physiologically relevant mechanism whereby [H$^+$] can control ET reactivity.[32−34] This effect is accessible in only certain cupredoxins and the structural features which influence it have remained elusive. In PAZ a limited decrease in the pK_a of His81 is observed in the Met16Phe and Met16Trp variants, whereas in both spinach and *P. laminosum* PCs a larger increase is found upon making the Leu12/14Phe mutations. In the Cu(I) Leu14Phe *P. laminosum* PC structures, His ligand protonation and dissociation is observed in two different crystal forms of the protein. In both, the imidazole of the "rotated" His92 conformer exhibits considerably better overlap with the phenyl group of Phe14 [the Phe14 C^ζ to His92 $C^{\varepsilon 1}$ distance in the protonated conformer is 0.4−0.7 Å shorter than the Phe14 C^ζ to His92 $N^{\varepsilon 2}$ distance in the coordinated form], with the two rings more parallel (see Figure 3). The positive charge on the imidazolium ion, along with enhanced overlap with π-electrons from the Phe, will stabilize this form and thus the pK_a of His92 increases upon the introduction of an adjacent aromatic side chain. The almost identical increase in the Leu12Phe spinach PC variant indicates that the introduced π-interaction in this protein has a similar effect. In the Met16Phe PAZ variant the altered overlap between the imidazole (His81) and phenyl (Phe16) rings (see Figure 6) must preferentially stabilize the coordinated (deprotonated) over the protonated state. However, it should not be overlooked that the removal of Met in PAZ, rather than Leu in PC could be responsible for the different effects observed on the His ligand pK_a (*vide infra*). The presence of a side-chain capable of participating in a π-interaction with a coordinating His can influence the ability of this ligand to protonate. An analogous interaction involving His18 in barnase and the native Trp at position 94, although with the two ring systems less parallel, has been found to stabilize the protonated form of the His, with a similar effect observed in the Trp94Phe and Trp94Tyr variants.[71] Likewise, a His-Phe contact stabilizes an Ala-based α-helix, again with the protonated His

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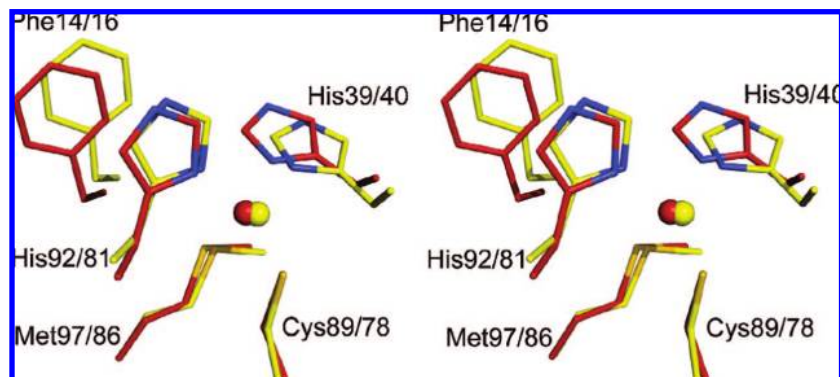(71) Loewenthal, R.; Sancho, J.; Fersht, A. R. *J. Mol. Biol.* **1992**, *224*, 759–770.

Figure 6. Stereoview showing an overlay of the Cu(II) site structures of Leu14Phe *P. laminosum* PC (yellow) and Met16Phe PAZ (dark red).

enhancing the effect.[72] A number of structural attributes have been suggested to influence His ligand protonation in cupredoxins,[11,12,15,30–32,36,47,48,69,73–77] yet this study is the first to identify such a feature.

In the case of *D. crassirhizoma* PC the relatively low pK_a for the coordinating His90 does not appear to be due to the presence of a π-interaction with Phe12 as mutation of this residue to a Leu has almost no effect.[35] The Gly36Pro mutation also has little influence on His90 protonation,[35] which is in agreement with the results obtained herein for the limited effect of the Pro36Gly mutation in spinach PC. Crystallographic studies on Cu(I) poplar PC highlighted that upon lowering the pH a large change in the conformation of Pro36 occurs.[32] It appears that a Gly in this position also provides the flexibility needed for the structural rearrangements that take place upon His protonation in PC [there are limited changes at Pro38 in the Cu(I) Leu14Phe structures but the adjacent Pro37 exists in two conformations in chain A of the pH 5.1 structure indicating flexibility in this region]. It is interesting to note that in the cupredoxin amicyanin, the corresponding Pro52Gly mutation results in a sizable decrease in the pK_a for the His95 ligand.[78] Therefore, the influence of the residue found at this position on the His ligand pK_a also varies in different members of the family. Surprisingly, the crystal structures of both Cu(I) WT[24] and Gly36Pro[35] *D. crassirhizoma* PC at low pH show no sign of His90 protonation, unlike poplar[32] and Leu14Phe *P. laminosum* PC (see Figure 3).

In this study we also find that π-interactions at the active site of an ET protein can affect other important physiological properties such as E_m and ET reactivity. The magnitude of this influence varies from protein to protein. In the case of PAZ significant increases in E_m are observed upon introducing Trp and Phe residues adjacent to the His81 ligand, and thus the Cu(I) forms of both variants are stabilized over the Cu(II) species. However, in spinach PC the introduction of a Phe adjacent to the coordinating His has almost

no effect on E_m, consistent with the limited influence (∼10 mV decrease) reported for the Phe12Leu mutation in *D. crassirhizoma* PC.[35] The significant stabilization of the Cu(I) forms of Met16Phe and Met16Trp PAZ may be due to the introduced π-interaction with the coordinating His altering the electronic structure of the copper site (this contact differs in the PAZ and PC variants, see Figure 6 and *vide supra*, and also varies in Met16Phe and Met16Trp PAZ). Alternatively, subtle three-dimensional structural alterations at and around the active site, including solvation differences, which are beyond the resolution of the crystallographic data presented here, may be responsible. What is particularly revealing from the overlay of the structures of Met16Phe and WT PAZ (see Figure 1) is the close overlap of the Met and Phe side chains. The orientation of Met16 is conserved in all published structures of WT and mutants of PAZ (including apoprotein) from a range of organisms.[6,53,59,79–81] This suggests that a well-defined site exists to accommodate the residue at position 16, and that the Met side chain is involved in a specific interaction with the His81 ligand in the WT protein. The equivalent position to Met16 in the cupredoxins azurin[10,82] and amicyanin[83] is also occupied by a Met, although in both cases the sulfur atom does not interact with the imidazole as in PAZ. The interactions made by the side chains of Met residues in proteins have been analyzed and favorable contacts with aromatic residues, including His, identified.[84–87] In PAZ the C^γ-S^δ-C^ε fragment of Met16 is coplanar with the coordinating His81 and the sulfur is directly above the imidazole ring (4.0 Å from the N^{δ1} of His81), and is only 3.7 Å from the C^{ε1} of His40 (also a ligand). Roles for Met residues in minimizing the effects of oxidative damage,[88] and facilitating protein interactions[89] (the dimer interface of azurin[82] which is the

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proposed homodimer structure for ESE[90,91] involves opposing Met side chains) is well recognized. The interaction of Met16 with His81 in PAZ will introduce rigidity to the Met side chain which may disfavor protein association (*vide infra*).[89] The Met-His interaction may therefore be more important for tuning the active site properties of this cupredoxin.

The effect of the introduced π-interaction on ET reactivity has been analyzed in PAZ. Met16 is in the hydrophobic patch of this cupredoxin, which is involved in the interaction with its physiological partner NIR.[42] Furthermore, Leu12/14 in spinach (and parsley)/*P. laminosum* PCs have been identified as components of the interface with cytochrome *f* and PSI,[38,39,92,93] which are its electron donor and acceptor respectively. The hydrophobic patch is also utilized for ESE in cupredoxins, an ET reaction which has no driving force and therefore provides a measure of intrinsic ET reactivity.[30,49,65,90,91,94,95] The introduction of a bulky aromatic side chain adjacent to the His81 ligand increases hydrophobicity in this region and is expected to enhance the association of two PAZ molecules [$k_{ESE} = k_{ET}$ (rate of ET) $\times K_{ESE}$ (the association constant for two PAZ molecules)]. Indeed k_{ESE} increases $\sim$2-fold in Met16Phe and $\sim$3-fold in the Met16Trp variant. The interaction between PAZ and NIR decreases 2-fold in Met16Phe, whereas a Trp at this position has little influence. k_{cat} for the Met16Phe and Met16Trp PAZ variants decreases approximately 2-fold, as a consequence of the decreased driving force for ET due to the higher E_m values for the mutants (the E_m for the T1 site of WT NIR, the initial port of entry of electrons into this enzyme, is 260 mV). The introduction of an aromatic residue in the hydrophobic patch of PAZ adjacent to the His81 ligand has a mainly subtle effect on ET reactions.

Conclusions

The studies reported herein find that the introduction of a π-interaction at the active site of an ET metalloprotein can alter E_m and also ET reactivity. This influence must result from either

subtle structural or electronic alterations. The pK_a of the His ligand involved in the π-interaction is regulated by this contact. The degree of π-overlap between an aromatic side chain in the second coordination sphere and a metal-bound imidazole influences whether the protonated or deprotonated (coordinated) form of the His is preferentially stabilized. This is the first identification of a structural feature that can control this potentially important physiological redox switch, which is accessible only in certain cupredoxins. A significant interaction from a second coordination sphere Met side chain with the His ligand is also identified that may contribute to the effect of the mutations made in PAZ.

Supporting Information Available: Detailed materials and methods and spectroscopic and electron self-exchange rate constant results, as well as Tables comparing the active site structures of WT and Met16Phe PAZ and also WT and Leu14Phe *P. laminosum* PC. Also included are figures showing UV/vis and EPR spectra, the dependence on pH of the chemical shift values of His ligand resonances in WT, Met16Phe and Met16Trp PAZ and also in WT and Leu14Phe *P. laminosum* PC, plots of T_1^{-1} against [Cu(II)] for His ligand resonances in Met16Phe PAZ and Lineweaver–Burk plots for the oxidation of Cu(I) WT, and Met16Phe and Met16Trp PAZ by NIR. This material is available free of charge via the Internet at http://pubs.ac.org.

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