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Review

# Investigating the structure and function of cupredoxins

Christopher Dennison *

*Institute for Cell and Molecular Biosciences, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, UK*

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## Contents



**Abstract**

Copper is widely used in nature to promote electron transfer in a variety of processes. The metal is usually found as a mononuclear type 1 copper site protected by a protein envelope, which has become known as a cupredoxin fold. In the past few years, the use of protein engineering combined with various spectroscopic and kinetic approaches has provided detailed information about cupredoxins and cupredoxin domains. This review will describe some of the recent advances that have been made, highlighting that there is still a long way to go before we fully appreciate the complexity of biological electron transfer proteins.


*Keywords:* Metalloproteins; Copper proteins; Cupredoxins; Phytocyanins; Electron transfer; Mutagenesis

## 1. Introduction

Nature utilises metals for a wide range of different biological functions. In the case of electron transfer (ET) iron and copper are employed due to their facile redox chemistry. Iron is found either associated with a porphyrin and a range of axial ligands in heme proteins [1–3] or as mono-, di-, tri- or tetra-nuclear sites possessing nearly always just sulfur ligands in the Fe–S proteins [1,2,4]. Copper centres, which promote biological ET are much less varied and occur mainly as either mononuclear type 1 (T1, also known as blue copper sites due to their intense colour) or dinuclear $Cu_A$ sites [5,6]. There are some exceptions such as the ET copper sites found in the enzyme peptidylglycine α-hydroxylating monooxygenase and probably also dopamine β-monooxygenase [7]. T1 copper and $Cu_A$ centres are found in a wide range of

* Tel.: +44 191 222 7127; fax: +44 191 222 7424.
*E-mail address:* christopher.dennison@ncl.ac.uk.
*URL:* http://www.ncl.ac.uk/camb/staff/profile/christopher.dennison.

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organisms and in all cases a very similar protein envelope protects the metal ion and tunes its reactivity, and is commonly referred to as a cupredoxin fold [8]. Cupredoxins can occur as either single domain proteins or as components of larger enzymes [9], such as in the copper-containing nitrite reductases (NiRs) which have a T1 site along with a catalytic type 2 (T2) copper centre [10,11], and the multi-copper oxidases including laccases (LACs) [12], ceruloplasmin [13], Fet3p [14] and ascorbate oxidase [15] [the multi-copper oxidases possess dinuclear type 3 (T3) and T2 coppers in close proximity giving the $Cu_3$ site]. Cytochrome *c* oxidase and nitrous oxide reductase bind $Cu_A$ centres via large cupredoxin domains [16,17]. Cupredoxin-like domains have also recently been found in proteins involved in copper homeostasis [18,19].

Cupredoxins have been investigated using a variety of structural, spectroscopic, kinetic and protein engineering approaches. More than ten distinct sub-families of cupredoxins have now been characterised (see Table 1). Although these proteins have been studied in detail many unanswered questions remain. For example, what is the exact physiological function of a number of the proteins listed in Table 1?

Table 1
Some properties of the known families of cupredoxins

| Protein (abbreviation) | Source | First isolated/ characterised | PDB code of first crystal structure[a] | $E_m$[b] (mV) | Physiological function |
|---|---|---|---|---|---|
| Azurin (AZ) | Bacteria | 1962 [20] | 1AZU[c] [21] | 310[c] [22] | Proposed role in ET related to the cellular response to redox stress [23] |
| Amicyanin (AMI) | Methylotrophic bacteria | 1981 [24] | 1MDA[d] [25] | 260[e] [26] | Electron acceptor from methylamine dehydrogenase [27] |
| Plastocyanin (PC) | Plant/alga | 1960 [28] | 1PLC (replaced lPCY)[f] [29] | 370[g] [30] | Photosynthetic ET between cytochrome *f* and P700 [31] |
| Pseudoazurin (PAZ) | Denitrifying bacteria and methylotrophs | 1973 [32] | 1PAZ[h] [33] | 280[i] [34] | Electron donor to nitrite reductase [35] |
| Rusticyanin (RST)[j] | Acidophilic bacterium[k] | 1975 [36] | 1RCY [37] | 670[l] [38] | Electron donor to cytochrome $c_4$ during Fe(II) oxidation [39] |
| Auracyanin (AUR)[j,m] | Photosynthetic bacterium[n] | 1992 [40] | 1QHQ[m] [41] | 240[m] [40] | Photosynthetic ET (proposed) [40] |
| Stellacyanin (STC)[o,p,q] | Plant | 1967 [45] | 1JER[r] [46] | 190[s] [22] | Unknown[t] |
| Plantacyanin (PLN)[o] | Plant | 1974 [51] | 2CBP (replaced 1CBP)[r] [52] | 310[r] [22] | Unknown[t] |
| Uclacyanin (UCA)[j,o,q] | Plant[u] | 1998 [53] | – | 320 [53] | Unknown[t] |
| Halocyanin (HAL)[j,q] | Haloalkaliphilic bacterium[v] | 1993 [54] | – | 220 [54] | Unknown |
| Sulfocyanin (SLF)[j,q] | Acidophilic bacterium[w] | 2001 [55] | – | 300 [55] | Electron donor to a terminal oxidase (proposed) |
| Nitrosocyanin (NTS)[j,x] | Autotrophic bacterium[y] | 2002 [56] | 1IBY [57] | 90 [56] | Proposed role in denitrification [58] |

[a] For the Cu(II) forms.
[b] To the nearest 10 mV and at ~neutral pH.
[c] From *Pseudomonas aeruginosa*.
[d] From *Paracoccus denitrificans*.
[e] From *Paracoccus versutus*.
[f] From poplar leaves.
[g] From spinach.
[h] From *Alcaligenes faecalis*.
[i] From *Achromobacter cycloclastes*.
[j] These cupredoxins have only been characterised from a single organism.
[k] From *Thiobacillus ferrooxidans*.
[l] At pH < 3.
[m] Auracyanin A and B have been characterised and the data listed here are for the B form.
[n] From *Chloroflexus aurantiacus*.
[o] The stellacyanins, plantacyanins and uclacyanins together form the phytocyanins.
[p] The proteins umecyanin (UMC) from horseradish roots [42,43] and mavicyanin (MAV) from zucchini [44] have been characterised and are considered to be stellacyanins.
[q] These cupredoxins are all thought to be cell wall/membrane anchored.
[r] From cucumber.
[s] From *Rhus vernicifera*.
[t] The transcript abundance of *bcb* (the gene for BCB, which is a STC from *Arabidopsis thaliana*) is enhanced under a variety of conditions which are known to induce oxidative stress [47–49]. As BCB is only one of about 50 phytocyanin sequences expected to be found in the *A. thaliana* genome a variety of functions is possible which are reviewed in Ref. [50].
[u] From *A. thaliana*.
[v] From *Natronobacterium pharaonis*.
[w] From *Sulfolobus acidocaldarius*.
[x] Nitrosocyanin possesses a T2 copper site and is a functional trimer but is included here due to it being made up of only three cupredoxin domains.
[y] From *Nitrosomonas europaea*.

What is the influence of natural variations in the coordination environment of cupredoxins on ET reactivity? Which surface attributes of cupredoxins are most important for interactions with physiological partners? Which features have the most significant influence on the reduction potential ($E_m$) of a T1 copper site? Which factors control the pH-induced active site alteration that dramatically influences the $E_m$ and ET reactivity of certain cupredoxins? In this review we will highlight the advances that have been made in recent years in addressing some of these questions and thus in developing our understanding of cupredoxins.

## 2. Cupredoxin structure

The first crystal structure of a cupredoxin was that of poplar plastocyanin (PC) which was determined in 1978 [29]. Numerous subsequent crystallographic studies on cupredoxins (see Table 1 [8,9,59–66]) have been published. The overall structure of a cupredoxin consists of β-strands arranged into two β-sheets forming a Greek key β-barrel structure—the cupredoxin fold (see Fig. 1). Variable amounts of α-helical secondary structure are present but this never contributes to the core of the protein. The copper ion is situated at one extreme of the molecule and is usually coordinated in a distorted tetrahedral fashion, with strong ligands provided by the thiolate sulfur of a Cys and the imidazole nitrogens of two histidines (see Fig. 2). The active site is typically completed by an unusually long bond to an axial Met ligand. The $His_2Cys$ equatorial ligand set is always maintained in cupredoxins and cupredoxin sub-domains, but the residue in the axial position can vary, as in the stellacyanins (STCs) where a Gln coordinates (see Fig. 2) [43,46], in some putative plantacyanins (PLNs) which have either a Val or Leu in the axial position [50,53,69,70], and also in fungal LACs, ceruloplasmin and Fet3p where a Phe or Leu is found in place of the Met [12–14,68,71]. The sites with a non-coordinating side-chain in the axial position have trigonal active site geometries (see Fig. 2). In the azurins (AZs) the backbone carbonyl oxygen

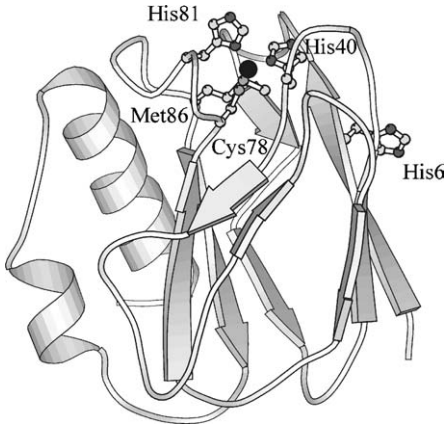



Fig. 1. The structure of Cu(II) PAZ from *A. cycloclastes* [60] (PDB accession code 1BQK) drawn with MOLSCRIPT [67]. The ligating amino acids and the surface His6 residue are included with the copper ion shown as a black sphere.

of a Gly residue provides a second weak axial interaction resulting in a trigonal bipyramidal geometry (see Fig. 2) [21,61–63]. The imidazole group of the C-terminal His ligand is always solvent exposed and surrounded by a surface hydrophobic patch which is important for interactions and ET with redox partners [25,72–74].

An intricate hydrogen bonding pattern exists around the copper centres of cupredoxins which is key to stabilising the active site structure [9]. The residue adjacent to the N-terminal His ligand, which is usually an Asn, is involved in a number of these interactions, with one of the key hydrogen bonds being between its backbone NH and the thiolate sulfur of the Cys ligand. Another hydrogen bond donor to the thiolate sulfur ligand is provided by the backbone NH of the residue found two after the Cys in certain cupredoxins. This second hydrogen bond is not found in those cupredoxins which have a Pro residue in this position {PC [59], amicyanin (AMI) [64,65] and pseudoazurin (PAZ) [60] for example}. AZ [61–63], and probably all phytocyanins [43,46,66,75], have this hydrogen bond which has been suggested to be

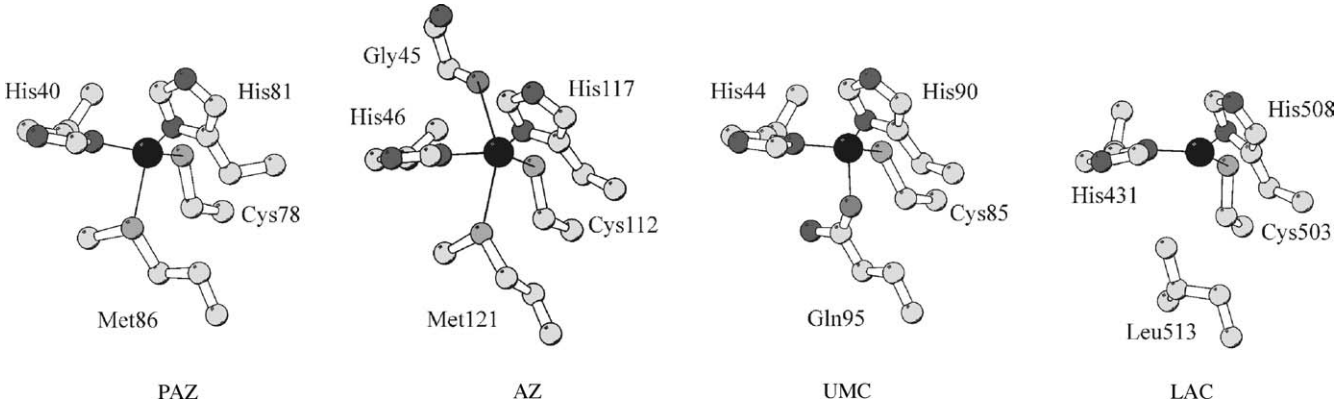



Fig. 2. Active site structures of Cu(II) PAZ from *A. cycloclastes* [60] (PDB accession code 1BQK), Cu(II) AZ from *P. aeruginosa* [63] (PDB accession code 1JZF), Cu(II) UMC from horseradish roots [43] (PDB accession code 1Z9R) and Cu(II) LAC from *Melanocarpus albomyces* [68] (PDB accession code 1GW0) drawn with MOLSCRIPT [67].

important for controlling various properties of their active sites [9,66].

The typical distorted tetrahedral T1 copper site structure found in cupredoxins is not the preferred geometry for either four-coordinate Cu(II), which tends to be square planar, or Cu(I), which usually has a more regular tetrahedral arrangement [76]. T1 copper sites undergo very small structural changes upon redox interconversion [60,63,77–80] and the active sites of metal substituted [81–85] and apo-proteins [64,86–89] are all very similar. Thus, the rigid cupredoxin domain holds the ligands in a particular arrangement giving a copper site geometry which is not optimal for either Cu(II) or Cu(I) and which does not change dramatically upon redox interconversion (an entatic [90] or rack-induced [91] state). Consequently, the reorganisation energy ($\lambda$) of T1 copper sites are small and are in the range of ∼0.7 eV [76,92,93], with the inner sphere reorganisation energy ($\lambda_i$), which originates from the ligands, thought to be as low as ∼0.3 eV [94,95]. The $\lambda$ values for small molecule copper complexes are considerably larger than this [76,92]. The results of calculations [96] have suggested that the ligands and geometry of a T1 copper centre are almost equally acceptable for Cu(II) and Cu(I) suggesting that the site is not entatic. More recent theoretical studies have indicated that the influence of the protein is much more significant [97].

## 3. Copper site properties

T1 copper sites possess unusual spectroscopic properties in their cupric states as a consequence of their coordination geometry. This includes an intense S(Cys)$\pi \rightarrow$ Cu(II)$d_{x^2-y^2}$ ligand-to-metal charge transfer (LMCT) transition at around 600 nm in their visible spectra ($\varepsilon \sim 2000$–$6000\ M^{-1}\ cm^{-1}$) [97–102]. A second LMCT transition [S(Cys)$\sigma \rightarrow$ Cu(II)$d_{x^2-y^2}$] at higher energy is observed and the intensities of these two bands vary in T1 sites [97,103–106] (this band is commonly referred to as being at ∼450 nm but the broad feature at this position can be made up of a number of transitions [97]). Electron paramagnetic resonance (EPR) spectroscopy has been used extensively for the investigation of T1 and $Cu_A$ centres [97,100–102,107–119]. EPR provides detailed information about biological copper sites and forms the basis of their classification (see Ref. [118] for an historical account of biological copper site classification). The spectra of T1 copper sites have unusually small hyperfine coupling constants in the $g_z$ region, which has been attributed to the highly covalent nature of the Cu–S(Cys) bond [101,102]. The difference between $g_x$ and $g_y$ in the EPR spectra of T1 copper sites can vary [97,103–106]. Similar $g_x$ and $g_y$ values give rise to an axial EPR signal whereas a larger separation between $g_x$ and $g_y$ results in a spectrum which is rhombic in appearance. The subtle variations in visible and EPR spectral properties of cupredoxins have lead to T1 copper sites been classified as either classic or distorted (perturbed) [97,105,106]. The classic sites have lower absorbance at ∼450 nm in their visible spectra and axial EPR features whereas increased 450 nm absorption and a rhombic EPR spectrum are attributes associated with distorted T1 sites. A correlation has been proposed between the length of the Cu–S(Cys) bond, the ratio between the intensities of the LMCT bands in the visible spectrum and the degree of rhombicity in the EPR spectrum of T1 centres [103,104]. More recently a coupled distortion model has assigned the properties of perturbed T1 sites to a shortening of the axial Cu–S(Met) interaction which leads to a weaker Cu–S(Cys) bond that collectively result in a tetragonal Jahn–Teller distortion [105,106]. This alteration is quantified by the angle between the $N_{His}CuN_{His}$ and $S_{Cys}CuS_{Met}$ planes, with a smaller value consistent with a perturbed site.

The $E_m$ values of cupredoxins are all higher than that of the Cu(II)/Cu(I) aqua couple (see Table 1) and if the T1 copper sites found in the multi-copper oxidases are included then a large range of values is found (the three-coordinate T1 sites of LACs and ceruloplasmin can have $E_m$ values as large as 800–1000 mV [22,97,120,121]). It is interesting to note that for the single domain cupredoxins almost all of the proteins have $E_m$ values which cover only a ∼200 mV range. The exception is rusticyanin (RST) which is unusual in that it is an acid stable cupredoxin. Thus, the popularly quoted range of $E_m$ values for cupredoxins is a little misleading with the majority having relatively similar values (190–370 mV). The axial ligand at a T1 copper site can tune $E_m$ over a range of ∼300 mV within a particular protein fold [97,122,123]. A number of other factors also play a significant role in controlling $E_m$ [76,124,125] including the active site structure which is dictated by the constrained protein, the hydrogen bonding pattern around the active site and the electrostatic environment of the protein.

## 4. Nuclear magnetic resonance studies of paramagnetic copper proteins

Nuclear magnetic resonance (NMR) spectroscopy has been utilised for many years to study cupredoxins. This has resulted in numerous solution structures of mainly the Cu(I) proteins [126–134] and also for a Cu(II) PC [135]. NMR has also been used to study localised structural changes (usually as a function of altering pH—see Section 5) and also the backbone dynamics of cupredoxins [133,134,136–147]. It was thought [148] that the relatively long electronic relaxation times of mononuclear Cu(II) sites in proteins (including cupredoxins) precluded the use of paramagnetic NMR to obtain information about their active sites (due to excessive broadening of the hyperfine shifted resonances). One elegant way around this problem was developed using Cu, Zn superoxide dismutase, where substitution of Zn(II) with Co(II) resulted in enhanced electronic relaxation at the Cu(II) site due to magnetic coupling, allowing relative sharp isotropically shifted resonances to be observed and assigned [148–150]. An alternative approach, used quite extensively

for cupredoxins, is to replace copper with a faster relaxing paramagnetic metal ion such as Co(II) and Ni(II) [151–175] (vide infra) whose spectra possess much sharper shifted resonances.

Approximately ten years ago paramagnetically shifted resonances for Cu(II) cupredoxins were observed for the first time. The initial studies were carried out on AMI, and utilising the electron self exchange (ESE) reaction correlations could be observed between paramagnetically shifted resonances and their diamagnetic counterparts in mixtures of Cu(II) and Cu(I) protein [176]. The observed shifts ($\delta_{obs}$) possess dipolar [pseudo-contact (pc)] and through bond [Fermi-contact (Fc)] contributions as shown in Eq. (1):

$$\delta_{obs} = \delta_{dia} + \delta_{pc} + \delta_{Fc} \quad (1)$$

where $\delta_{dia}$ is the observed chemical shift in a corresponding diamagnetic system. The $\delta_{pc}$ values can be determined from the structure of the protein and the orientation of the magnetic susceptibility tensor. Due to the small anisotropy of the g tensor in Cu(II) cupredoxins the $\delta_{pc}$ values are not large [the $\delta_{pc}$ contributions are more sizable in the Co(II) and Ni(II) substituted proteins (vide infra)] and the isotropic shifts ($\delta_{iso} = \delta_{pc} + \delta_{Fc}$) are mainly composed of $\delta_{Fc}$. These $\delta_{Fc}$ shifts provide the hyperfine coupling constants which are a quantitative measure of the spin density distribution over the copper ligands and hence give detailed information about the geometric and electronic structure of the active site.

The active sites of Cu(II) PCs from various sources [135,177,178], AZ [179], cucumber STC (CST) [179], PAZ [144,180,181], RST [181] and the STC from horseradish roots (umecyanin, UMC) [175] have been studied in detail by paramagnetic NMR spectroscopy (see Fig. 3 and Table 2). Saturation transfer experiments on mixtures of Cu(II) and Cu(I) proteins have been used to assign the paramagnetic NMR spectra (see Fig. 4). For spinach and *Synechocystis* PCs, AZ, CST, PAZ and RST "blind" saturation transfer experiments were used to investigate signals broadened beyond detection. This included the Cys $C^{\beta}H$ proton resonances whose $\delta_{Fc}$ values provide detailed information about the interaction of this important ligand with Cu(II). A Karplus-type relationship with a $\sin^2\theta$ dependence of the $\delta_{Fc}$ values of these signals on the Cu–$S^{\gamma}$–$C^{\beta}$–$H^{\beta}$ dihedral angles was proposed for PC [177], suggesting a mainly π-type delocalisation mechanism (a σ-type distribution mechanism would lead to a $\cos^2\theta$ dependence) [182]. This is consistent with the dominant π-overlap between the Cu $d_{x^2-y^2}$ and S(Cys)π orbitals at the classic T1 copper site of PC [97]. The absence of stereospecific assignments for the Cys $C^{\beta}H$ proton resonances in the other cupredoxins studied has prevented further analysis, particularly at distorted T1 sites. The average chemical shift of the $C^{\beta}H$ protons of the coordinated Cys ($\delta_{\beta,av}$) is almost independent of the conformation of this ligand and the values observed for cupredoxins cover a large range (see Table 2). In the spectrum of AZ [179] the Cys $C^{\beta}H$ protons exhibit the largest $\delta_{\beta,av}$ value (825 ppm) with the smallest

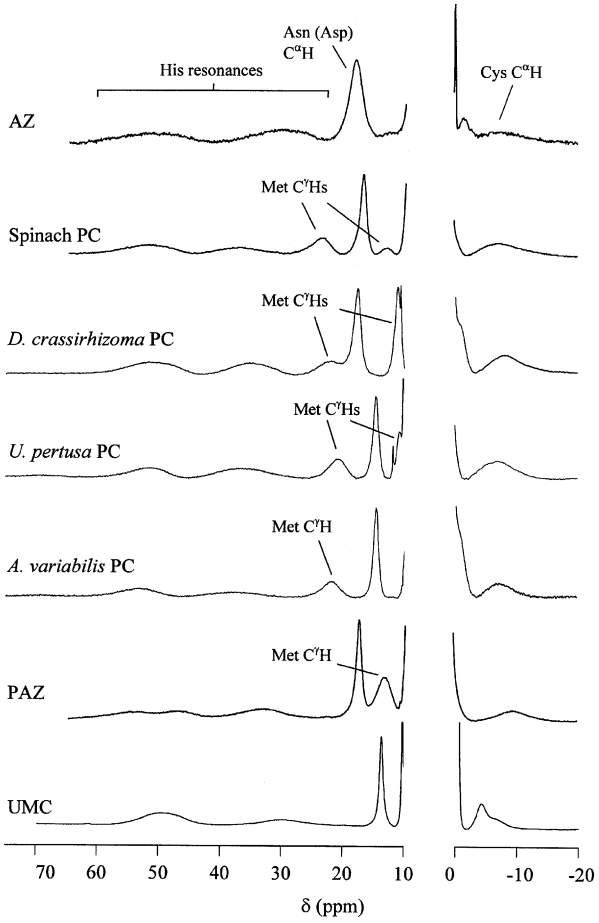



Fig. 3. $^1$H NMR spectra of Cu(II) cupredoxins (500 MHz) in 99.9% deuterated phosphate buffer all at pH* 8.0 (pH* indicates a pH meter reading uncorrected for the deuterium isotope effect) except for those of PAZ and UMC which were obtained at pH* 7.6. All of the spectra were acquired at 25 °C except for those of *Dryopteris crassirhizoma* PC and UMC which were obtained at 30 and 40 °C, respectively.

$\delta_{\beta,av}$ found for RST (270 ppm) [181]. There is a general decrease in the $\delta_{\beta,av}$ value, indicating a weaker Cu(II)–S(Cys) bond, as the axial Cu(II)–S(Met) interaction increases and the T1 site becomes more distorted (the paramagnetic NMR spectra of cupredoxins with axial Gln ligands will be discussed in Section 7). It has been suggested that the $\delta_{\beta,av}$ values depend on the Cu(II)–S(Cys) bond length and the angle between the $CuN_{His}N_{His}$ plane and the Cu–S(Cys) vector [181].

Paramagnetic NMR has been used to assess the differences between the Cu(II) sites of one family of cupredoxins (the PCs, see Fig. 3 and Table 2) obtained from different sources [178]. The similarity of the $\delta_{obs}$ (and therefore $\delta_{Fc}$) values for the His ligands indicates very similar spin densities on these ligands in the PCs [the His ligand resonances are actually found at very similar positions in the spectra of all Cu(II) cupredoxins]. This is particularly noteworthy for the *Dryopteris crassirhizoma* (a fern, i.e. a seedless vascular plant) PC which has a novel π–π contact between the imidazole ring of the His90 (His87 in other PCs) ligand

Table 2
The assigned hyperfine shifted resonances in the $^1$H NMR spectra of various Cu(II) cupredoxins

| $\delta_{obs}$ (ppm) in Cu(II) cupredoxins | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Proton[a] | AZ[b] | AMI[c] | PCs[d] | | | | | | |
| | | | Spinach[e] | *D. crassirhizoma*[f] | *U. pertusa*[g] | *Synechocystis*[h] | PAZ[i,j] | CST[k] | UMC[l] |
| His $C^{\delta 2}H$ | 49.1 | 43 | 47.1 | 48.2 | 50.2 | 51.1 | 46 | 55.0/48.0 | 51/48 |
| His $C^{\varepsilon 1}H$ | 46.7/34.1 | ∼30 | 35.6 | 34.9 | 38.0/34.3 | 35.7 | 33 | 41.2/29.8 | ∼30 |
| His $N^{\varepsilon 2}H$ | 26.9 | 27.5 | 31.4 | 31.2 | 33.7 | 31.1 | 22.8 | 26 | 28.5[m] |
| Cys $C^{\beta 1}H$ | 850/800 | | 650 | | | 614 | 510/390[n] | 450/375 | |
| Cys $C^{\beta 2}H$ | 850/800 | | 489 | | | 517 | 510/390[n] | 450/375 | |
| Cys $C^{\alpha}H$ | −7.0 | −9.5 | −8.0 | −8.0 | −7.5 | −7.8 | −9.7 | −7.5 | −6 |
| His $C^{\delta 2}H$ | 54.0 | 50 | 51.6 | 51.7 | 52.1 | 52.6 | 54 | 55.0/48.0 | 51/48 |
| His $C^{\varepsilon 1}H$ | 46.7/34.1 | ∼30 | 35.6 | 34.9 | 38.0/34.3 | 38.5 | 33 | 41.2/29.8 | ∼30 |
| His $N^{\varepsilon 2}H$ | 27 | | | 44.3[o] | 40.6[g] | 42.9 | | | ∼47[l] |
| Met $C^{\gamma 1}H$ | | 12.0/11.1 | 13.0 | 11.0 | 10.6 | | 13.0 | [p] | [p] |
| Met $C^{\gamma 2}H$ | | 12.0/11.1 | 23.5 | 22.3 | 20.7 | 24.0 | | [p] | [p] |
| Asn/Asp $C^{\alpha}H$ | 19.9 | 14.1 | 17.0 | 17.6 | 14.4 | 14.7 | 17.3 | 16.9 | 13.7 |

[a] From top to bottom: His46, Cys112, His117, Met121 and Asn47 for AZ; His54, Cys93, His96, Met99 and Asn55 for AMI; His37, Cys84, His87, Met92 and Asn38 for the PCs (small sequence differences exist between the PCs which alters ligand numbering); His40, Cys78, His81, Met89 and Asn41 in PAZ; His46, Cys89, His94 and Asn47 for CST; His44, Cys85, His90 and Asp45 for UMC.
[b] From *P. aeruginosa* measured at 5 °C and pH 8.0 [179].
[c] From *P. versutus* measured at 32 °C and pH 7.0 [176].
[d] From a variety of sources.
[e] At 25 °C and pH 7.5 [177].
[f] At 30 °C and pH 8.0 [178].
[g] At 25 °C and pH 8.0 [178].
[h] At 22 °C and pH 5.2 [135].
[i] From *A. cycloclastes* at 25 °C and pH 7.6 [144].
[j] The data for RST from *T. ferrooxidans* is very similar to that for PAZ except that the Cys $C^{\beta}H$ proton resonances are found at 300 and 240 ppm [181].
[k] STC from cucumber at 28 °C and pH 6.0 [179].
[l] STC from horseradish roots at 40 °C and pH 7.6 [175].
[m] At 25 °C and pH 4.6.
[n] At pH 8.0 [181].
[o] At 30 °C and pH 5.0.
[p] CST and UMC possess an axial Gln in place of Met and no shifted resonances arising from this ligand are observed.

and Phe12 (vide infra) [183,184]. However, this interaction does not seem to affect the spin density distribution onto this ligand. The Cu(II)–S(Cys) interaction is alike in all of the PCs whereas the axial Met signals show the largest differences. In all of the PCs the Met92 $C^{\gamma 2}H$ proton resonance is observed at ∼26–20 ppm. The Met92 $C^{\gamma 1}H$ proton signal is only shifted outside of the diamagnetic envelope in the higher plant (spinach and parsley), *D. crassirhizoma*, and *Ulva pertusa* (green alga) PCs and is not observed in the cyanobacterial (*Synechocystis*, *Synechococcus* and *Anabaena variabilis*) proteins. There does not appear to be a direct correlation between the Cu(II)–S(Met) bond lengths or the Cu–$S^{\delta}$–$C^{\gamma}$–$H^{\gamma}$ dihedral angles and the $\delta_{Fc}$ values of the Met $C^{\gamma}H$ proton resonances in the PCs [135,183–187]. It is interesting to note that Met $C^{\gamma}H$ proton resonances are only observed in AMI and PAZ of the other cupredoxins studied, and in both cases these signals do not experience particularly large isotropic shifts [144,176]. Therefore, the $\delta_{Fc}$ values of the $C^{\gamma}H$ proton resonances of the axial Met ligand do not seem to be influenced by the presence of a classic (PC and AMI) or a distorted (PAZ) T1 site. The comparative paramagnetic NMR studies of the PCs show that there is a close homology between the spin density distribution onto the ligands in these proteins, and the likeness is particularly high for those PCs from similar sources. However, the observed differences are not particularly large showing that the active site structures of Cu(II) PCs are alike and have not been altered significantly during the evolutionary process [178].

Although paramagnetically shifted resonances can be observed for Cu(II) cupredoxins the relatively slow electronic relaxation (and consequent signal broadening) of the native metal prevents direct observation of all active site resonances, and the analysis of metal substituted forms can be useful. The Co(II) and Ni(II) derivatives of cupredoxins were initially prepared to obtain active site information from their electronic spectra [151–153]. Initial paramagnetic NMR spectra were reported for Co(II) and Ni(II) AZ in 1976 [154] and 1982 [155], respectively. More detailed studies of these substituted forms of AZ followed [156–160,165,168] and various other Co(II) and Ni(II) cupredoxins have now been investigated with this approach, including a number of active site variants [161–164,167,169–175]. The spectrum of Ni(II) PAZ has been assigned (the first study of a Ni(II) cupredoxin with a distorted T1 site) [172] allowing a detailed comparison of the available data for Ni(II) cupredoxins (see Table 3). The $\delta_{obs}$ values for Ni(II) cupredoxins possess larger $\delta_{pc}$ contri-

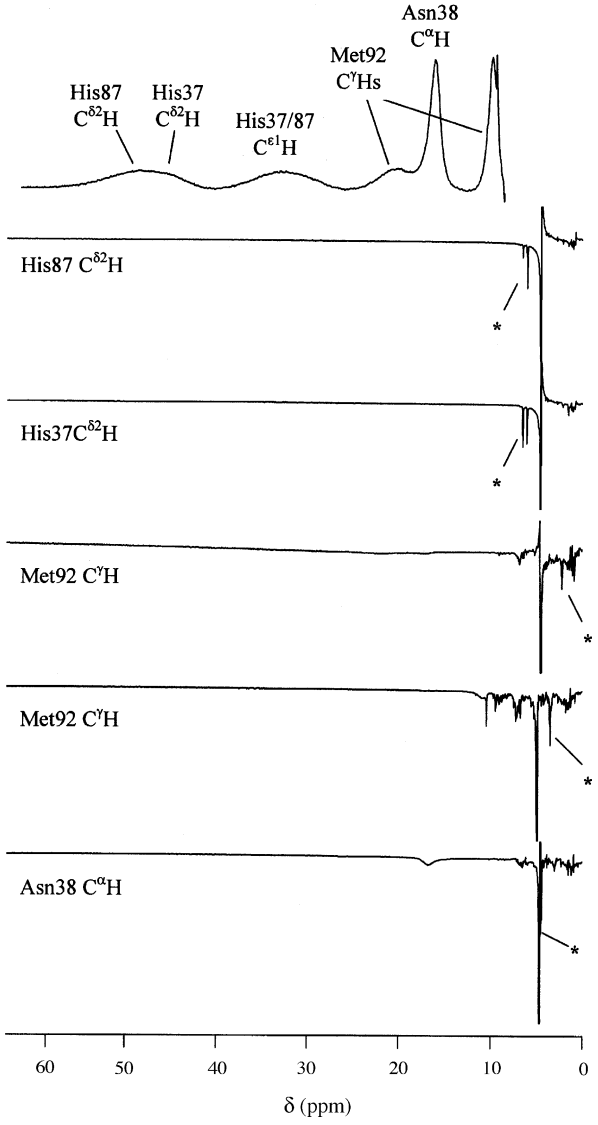



Fig. 4. $^{1}$H NMR saturation transfer difference spectra (30 °C) of a 1:1 mixture of *D. crassirhizoma* Cu(I) and Cu(II) PC in 35 mM phosphate buffer (99.9% $D_2O$) at pH* 8 [178]. The top spectrum is that of the Cu(II) protein and those below are the saturation transfer difference spectra in which the peaks indicated were irradiated. The observed saturation transfer peaks in Cu(I) PC are shown by an asterisk.

butions than the Cu(II) forms. These have been determined for Ni(II) AZ (see Table 3) and are actually not that significant so that $\delta_{obs}$ values can be compared, with caution, to provide comparative active site information. In these spectra the Cys $C^{\beta}H$ proton resonances are directly observed and their relatively small shifts indicate that the spin density on this ligand is considerably less in the Ni(II) cupredoxins than in the Cu(II) proteins (see Tables 2 and 3). The data for the substituted forms do not seem to provide a good relative indication of the covalency of the Cu–S(Cys) interaction as the $\delta_{\beta,av}$ value is smallest for Ni(II) AZ whereas the Cu(II) protein has by far the largest $\delta_{\beta,av}$ [sizable $\delta_{pc}$ contributions for the Cys $C^{\beta}H$ protons in Ni(II) AMI and PAZ could be a factor]. The $\delta_{\beta,av}$ values are similar for both Ni(II) PAZ [172] and Ni(II) AMI [169] which have distorted and classic T1 sites, respectively. The average of the $\delta_{obs}$ values of the $C^{\gamma}H$ protons of the axial Met ligand are 269, 101 and 56 ppm, which are consistent with the observed Cu(II)–S(Met) bond distances of 2.71, 2.84 and 3.32 Å, respectively, in the crystal structures of Cu(II) PAZ [60], AMI [65] and AZ [63]. Thus, the Ni(II) derivatives appear to provide reasonably accurate information about the relative strength of the axial interaction in cupredoxins (it is interesting to note that the Ni(II)–S(Met) bond distance is also ∼3.3 Å in Ni(II) AZ [84]).

The recent analysis of the paramagnetic NMR spectra of Co(II) PC [174] and Co(II) PAZ [173] has allowed a detailed comparison to earlier work on the same derivatives of AZ [158,159,165], AMI [169] and RST [171] (see Table 4). Again caution is required when interpreting the $\delta_{obs}$ values as sizable $\delta_{pc}$ contributions can be involved. These have been determined for both Co(II) AZ [165] and Co(II) RST [171] and demonstrate that the $\delta_{pc}$ values for the Cys $C^{\beta}H$ proton resonances can vary considerably. The $\delta_{obs}$ values for the Cys $C^{\beta}H$ protons in Co(II) RST (see Table 4), and most likely also Co(II) PC, Co(II) AMI and Co(II) PAZ possess larger $\delta_{pc}$ contributions than in Co(II) AZ, and thus the spin density on the Cys ligand is probably greatest for AZ [which does correspond with the data for the Cu(II) proteins (see Table 2)]. The Co(II)–S(Cys) bond, like that of the Ni(II) protein, is much less covalent than the Cu(II)–S(Cys) bond, and the relative $\delta_{\beta,av}$ values generally do not match those observed for the Cu(II) proteins (compare Tables 2 and 4). The Co(II)–S(Cys) and Co(II)–S(Met) interactions are similar in PC, PAZ and RST (see Table 4) and thus there appears to be very little difference in these metal–ligand contacts between the Co(II) derivatives of cupredoxins possessing a classic T1 site (PC) and those having perturbed features (PAZ and RST) (these three cupredoxins do possess quite similar Cu(II)–S(Met) distances). The spectrum of Co(II) AMI has some unusual features which could be due to the presence of a water molecule coordinated to the non-native metal [188] or the altered conformation of its axial Met ligand.

Paramagnetic NMR has also been applied to larger enzymes which possess cupredoxin sub-domains. Copper-containing NiRs, which function in the dissimilatory denitrification pathway of certain micro-organisms reducing nitrite to nitric oxide, have been studied [189]. NiRs are trimeric with each subunit having two cupredoxin-like domains but with only one of these possessing a T1 copper site [10,11,190]. There are three inter-subunit T2 copper centres (sites of nitrite binding) in close proximity to the T1 sites (12.5 Å away, see Fig. 5). NiRs can be either green (*Achromobacter cycloclastes* NiR) [11] or blue (*Alcaligenes xylosoxidans* NiR) [190] with the distinction due to the presence of either a distorted (the T1 site of green NiRs is the most distorted known) or a classic T1 copper site [97,105]. Hyperfine shifted resonances can be observed for NiRs and the spectra of the *A. cycloclastes* protein and the corresponding T2 depleted form (T2D, i.e. with copper removed from

Table 3
The observed hyperfine shifts ($\delta_{obs}$) in the $^1$H NMR spectra of Ni(II) cupredoxins

| Proton[a] | Ni(II) AZ[b] | | | Ni(II) AMI[c] | Ni(II) PAZ[d] | Ni(II) UMC[e] | Ni(II) STC[f] |
|---|---|---|---|---|---|---|---|
| | $\delta_{obs}$ (ppm) | $\delta_{pc}$ (ppm) | $\delta_{Fc}$ (ppm) | $\delta_{obs}$ (ppm) | $\delta_{obs}$ (ppm) | $\delta_{obs}$ (ppm) | $\delta_{obs}$ (ppm) |
| His $C^{\delta2}H$ | 57.2 | −0.3 | 51.9 | 72.6 | 71.0 | 52.5 | 52.1 |
| His $C^{\varepsilon1}H$ | 61.2 | 17.0 | 37.3 | 68/34 | 80.0 | ∼34 | 39.8/28.0 |
| His $N^{\varepsilon2}H$ | 35.0 | 2.0 | 21.5 | 38.3 | 38.5 | 57.4[g] | 50.8 |
| Cys $C^{\beta1}H$ | 187.0 | 1.1 | 182.5 | 296 | 274 | 167 | 177 |
| Cys $C^{\beta2}H$ | 233.0 | −9.8 | 239.5 | 254 | 297 | 224 | 197 |
| His $C^{\delta2}H$ | 64.3 | 1.6 | 55.9 | 58.3 | 48.7 | 69.5 | 67.1 |
| His $C^{\varepsilon1}H$ | 52.6 | 9.7 | 36.1 | 68/34 | | 39.5 | 39.8/28.0 |
| His $N^{\varepsilon2}H$ | 51.5[h] | 3.7 | 36.2 | | 43.2[i] | 39.5[g] | 33.9 |
| Met $C^{\gamma1}H$ | 111.3 | −2.7 | 112.5 | 172.5 | 432.5 | | |
| Met $C^{\gamma2}H$ | 0.6 | −4.9 | 4.0 | 30.2 | 105.4 | | |
| Met $C^{\varepsilon}H_3$ | 30.3 | −0.1 | 111.0 | 111.0 | 119.0 | | |
| Gln $C^{\gamma1}H$ | | | | | | 42.2 | 33.2 |
| Gln $C^{\gamma2}H$ | | | | | | −25.1 | −21.0 |
| Gln $N^{\varepsilon21}H$ | | | | | | −17.8 | −18.0 |
| Gly $C^{\alpha1}H$ | 69.5 | −6.5 | 65.4 | | | | |
| Gly $C^{\alpha2}H$ | 14.1 | −5.9 | −11.4 | | | | |

[a] From top to bottom: His46, Cys112, His117, Met121 and Gly45 for AZ; His54, Cys93, His96 and Met99 for AMI; His40, Cys78, His81 and Met86 for PAZ; His44, Cys85, His90 and Gln95 for UMC; His46, Cys89, His94 and Gln99 for CST.
[b] From *P. aeruginosa* at 45 °C and pH 7.5 including $\delta_{pc}$ and $\delta_{Fc}$ contributions [165].
[c] From *P. versutus* at 30 °C and pH 7.5 [169].
[d] From *A. cycloclastes* at 25 °C and pH 7.5 [172].
[e] The STC from horseradish roots at 30 °C and pH 8.0 [175].
[f] From *R. vernicifera* at 40 °C and pH 4.0 [167].
[g] At 2 °C and pH 5.6.
[h] Observed below pH 7.
[i] At 10 °C and pH 5.6.

Table 4
Comparison of the observed hyperfine shifts ($\delta_{obs}$) in the 1H NMR spectrum of Co(II) cupredoxins

| Proton[a] | Co(II) AZ[b] | | | Co(II) PC[c] | Co(II) AMI[d] | Co(II) PAZ[e] | Co(II) RST[f] | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\delta_{obs}$ (ppm) | $\delta_{pc}$ (ppm) | $\delta_{Fc}$ (ppm) | $\delta_{obs}$ (ppm) | $\delta_{obs}$ (ppm) | $\delta_{obs}$ (ppm) | $\delta_{obs}$ (ppm) | $\delta_{pc}$ (ppm) | $\delta_{Fc}$ (ppm) |
| His $C^{\delta2}H$ | 50.6 | 7.8 | 37.3 | 55.8 | 52.6 | 53.1 | 59.7 | −1.9 | 53.6 |
| His $C^{\varepsilon1}H$ | 97 | 85.7 | 4.4 | 133 | 118/38 | 57/146 | – | – | – |
| His $N^{\varepsilon2}H$ | 74.9 | 16.6 | 46.9 | 63.2 | 62.3 | 61.9 | 69.2 | 10.3 | 44.2 |
| Cys $C^{\beta1}H$ | 232 | 5.4 | 223.2 | 299 | 285 | 315/267 | 287/260 | 69.9 | 205 |
| Cys $C^{\beta2}H$ | 285 | −5.3 | 287.4 | 275 | 285 | 315/267 | 287/260 | 59.7 | 184 |
| His $C^{\delta2}H$ | 56.4 | 7.2 | 42.3 | 43.8 | 51.0 | 43.6 | 48.7 | 3.4 | 38.5 |
| His $C^{\varepsilon1}H$ | 75 | 23.3 | 45 | 60 | 118/38 | 57/146 | – | – | – |
| His $N^{\varepsilon2}H$ | 65.8 | 7.5 | 46.8 | 75.3[g] | 74[h] | 71.4 | 80.3 | 19.9 | 48.9 |
| Met $C^{\beta1}H$ | −18.9 | −25.9 | 5.3 | −18.4 | −18.6 | −31.2 | −31.0 | −23.2 | −9.8 |
| Met $C^{\beta2}H$ | −18.5 | −26.9 | 6.1 | −27.5 | −16.1 | – | −24.4 | −13.5 | −17.4 |
| Met $C^{\gamma1}H$ | 45.3 | −19.5 | 64.4 | 254 | 132.5 | 105.8 | 122.9 | −11.5 | 132.5 |
| Met $C^{\gamma2}H$ | −19.1 | −20.2 | −0.3 | 87.8 | 10.0 | 271.3 | 285.2 | −19.2 | 301.6 |
| Met $C^{\varepsilon}H_3$ | −7.3 | −31.2 | 24 | 80.4 | 74.5 | 90.2 | 103.3 | −16.9 | 130.3 |
| Gly $C^{\alpha1}H$ | 47.8 | −10.5 | 54.2 | | | | | | |
| Gly $C^{\alpha2}H$ | −29.4 | −26.7 | −5.9 | | | | | | |

[a] From top to bottom: His46, Cys112, His117, Met121 and Gly45 for AZ; His37, Cys84, His87 and Met92 for PC; His54, Cys93, His96 and Met99 for AMI; His40, Cys78, His81 and Met86 for PAZ; H His85, Cys138, His 143 and Met148 for RST.
[b] From *P. aeruginosa* at pH 4.5 and 37 °C [158,159,165] including $\delta_{pc}$ and $\delta_{Fc}$ contributions.
[c] From spinach at 30 °C and pH 7.8 [174].
[d] From *P. versutus* at 40 °C and pH 8.0 [169].
[e] From *A. cycloclastes* at 40 °C and pH 8.0 [173].
[f] From *T. ferrooxidans* at pH 6.0 and 20 °C including $\delta_{pc}$ and $\delta_{Fc}$ contributions [171].
[g] At 5 °C and pH 7.0.
[h] At 22 °C and pH 5.0.

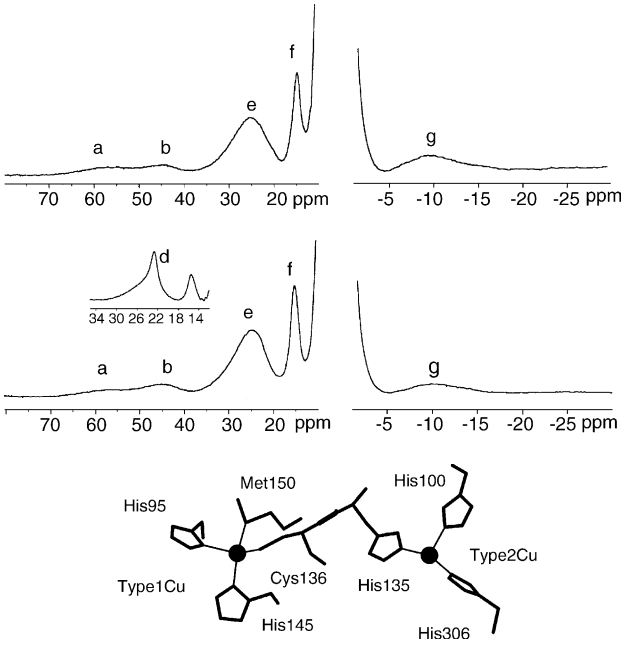



Fig. 5. Structure (bottom) of the Cu(II) sites in one of the monomers of the green NiR from *A. cycloclastes* [11] (PDB accession code 1NIB). The His306 ligand of the T2 copper centre originates from the adjacent monomer. Also shown are $^1$H NMR spectra of *A. cycloclastes* NiR (500 MHz, 25 °C): T2D protein in 20 mM phosphate in 99.9% $D_2O$ at pH* 7.5 with the inset showing part of the spectrum in 90% $H_2O$/10% $D_2O$ at pH 7.5 (middle). Fully copper loaded protein in 20 mM phosphate (99.9% $D_2O$) at pH* 7.0 (top). Assignments are: (a) and (b) His95/His145 $C^{\delta2}H$ protons; (d) His95 $N^{\delta2}H$, (e) Met150 $C^{\gamma}H$; (f) Asn96 $C^{\alpha}H$; (g) Cys136 $C^{\alpha}H$ [189].

the T2 centres) are almost identical (see Fig. 5). This demonstrates that all of the observed proton resonances arise from the T1 copper ligands. The resonances have been tentatively assigned (see Fig. 5) based on comparisons to the spectra of cupredoxins, as have those for the blue NiR from *A. xylosoxidans* [189]. There does appear to be differences in the $\delta_{Fc}$ values for the observed $C^{\gamma}H$ proton of the axial Met ligand even though the Cu–S(Met) bond lengths are very similar in the green (2.56 Å) and blue (2.64 Å) NiRs.

In order to further study the T1 site differences between green and blue NiRs, and also to investigate the T2 sites of these enzymes, paramagnetic NMR studies have been extended to their Co(II) substituted forms in our lab. In Fig. 6 the NMR spectra of *A. cycloclastes* NiR with Co(II) at both active sites [T1Co(II)/T2Co(II)] and also with Co(II) at just the T2 centre and Cu(I) at the T1 site [T1Cu(I)/T2Co(II)] are shown. Relatively sharp shifted resonances from both of the sites can be observed and many have been assigned. The same derivatives of *A. xylosoxidans* NiR are currently being studied to enable a detailed comparison of the active sites of blue and green NiRs [191].

Multi-copper oxidases are also proving amenable to study by paramagnetic NMR and attention has primarily focussed on LACs. These proteins possess a trinuclear $Cu_3$ cluster (made up of a T2 and a T3 centre in close proximity) along with a T1 site [12,13,15,68,71], which in certain fungal LACs possess a trigonal coordination geometry (no axial ligand) [12,68,71]. The paramagnetic NMR spectrum of the LAC from *Rhus vernicifera* (which from sequence alignments has an axial Met ligand at its T1 site) contains hyperfine shifted resonances arising from only the T1 site [192]. In the case of the novel small LAC from *Streptomyces coelicolor* (SLAC) shifted resonances from the $Cu_3$ centre are also identified [193]. Isotropically shifted resonances have been observed in spectra of the fungal LAC from *Polyporous versicolor* and can be assigned to both the T1 and the $Cu_3$ cluster [194].

Paramagnetic NMR investigations of copper proteins is an area which is still developing. Most of the cupredoxin subfamilies have now been studied with this approach and the spectra provide detailed information about active site structure. More work is required to understand the differences observed in the spectra of the Cu(II) proteins, and in particular for the Cys and Met resonances. Co(II) and Ni(II) substituted cupredoxins provide complimentary data which has to be interpreted with care. However, these investigations can provide detailed comparative information about aspects of T1 copper site structure and can identify ligating residues for proteins whose structures have not been determined (vide infra) [161,175]. Paramagnetic NMR is extremely useful in providing a rapid method for determining the influence of mutations on the active site structure of cupredoxins [either in their native Cu(II) forms or as metal substituted derivatives]. Furthermore, it is becoming clear that this technique can be applied to larger multi-copper enzymes and that other

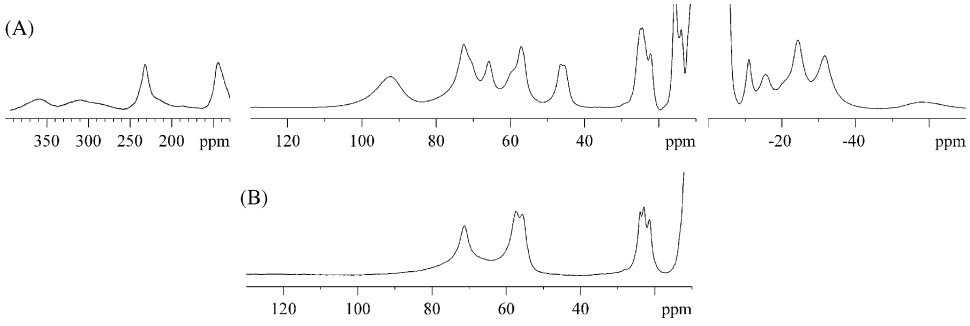



Fig. 6. $^1$H NMR spectra of the Co(II) substituted NiR from *A. cycloclastes* (300 MHz, 40 °C): (A) T1Co(II)/T2Co(II) protein in 10 mM phosphate in 90% $H_2O$/10% $D_2O$ at pH 8.0; (B) T1Cu(I)/T2Co(II) NiR in 10 mM phosphate in 90% $H_2O$/10% $D_2O$ at pH 8.0 [191].

copper centres as well as T1 and T2 sites can be investigated (the $Cu_A$ centre [195–199] and also the T3 site of tyrosinase [200–202] have been studied).

## 5. C-terminal histidine ligand protonation

It has long been recognised that amino acid residues, whose p$K_a$ values are in the accessible pH range, can have a significant effect on the structure and reactivity of cupredoxins [30,45,77]. For example it has been shown in PAZ that the protonation/deprotonation of the surface residue His6 results in an altered active site structure (the relative intensities of the LMCT bands are affected), influences the ESE reactivity (vide infra) and tunes the $E_m$ of the protein even though this residue is ~15 Å from the copper (see Fig. 1) [144]. Quantum chemical calculations have demonstrated that the influence of the protonation of His6 on the $E_m$ of PAZ is mainly electrostatic and the structural rearrangement does not significantly alter this parameter [203]. The protonation of the surface residues His35 and His83 in *Pseudomonas aeruginosa* AZ influence the $E_m$ of this protein [204] with the effect also being mainly electrostatic in nature [205] (the protonation of neither residue significantly alters the spectroscopic properties of the protein). The alkaline transition of phytocyanins, which influences their active site structure and reactivity, is caused by the protonation/deprotonation of a surface residue and will be discussed in Section 7.

Active site protonation occurs in certain reduced cupredoxins at the C-terminal His ligand (whose imidazole ring is exposed at the centre of the hydrophobic patch) and its influence is dramatic [143]. In PC [77], AMI [80] and PAZ [79] crystallographic studies have demonstrated that this His ligand dissociates from the cuprous ion at low pH resulting in a three-coordinate site. This effect has a large influence on the $E_m$ [206–208] and ET reactivity [178,209] and has also been demonstrated to occur in certain STCs [208,210] and PLTs [208,211]. The p$K_a$ value for the His ligand differs in cupredoxins with values of 6.7, 4.9, 4.9, 4.5, 4.3, 5.7 and 4.3 for AMI [138], PC [136,178], PAZ [139,212,213], *R. vernicifera* STC [208], the STC from zucchini (mavicyanin, MAV) [210], the PLT from spinach (PLN) [211] and the PLT from cucumber (cucumber basic protein, CBP) [208], respectively. Given the pH at which this effect usually occurs it has been called the acid transition [208]. In the case of AZ, whose His ligand does not protonate in the accessible pH range, a p$K_a$ of <2 has been calculated [214]. The protonation of this C-terminal His ligand occurs at much lower pH values in Cu(II) cupredoxins and an upper limit of 3.4 has been reported for the p$K_a$ of His96 in Cu(II) AMI [215]. A simple theory put forward some years ago [212] implicated the length and structure of the C-terminal ligand-containing loop, which runs from the Cys to the Met ligand and contains the exposed His ligand (see Fig. 7), as a major factor in controlling the acid transition of cupredoxins. It was noted that there were only two intervening residues between the Cys and His ligands in the proteins which, at that time, had been shown to exhibit the acid transition. The gap between the His and Met ligands seemed to correlate with the p$K_a$ value, with the shortest loop, and highest p$K_a$, being found in AMI. Loop-directed mutagenesis experiments in which the AMI loop was extended (loop-elongation) result in a lower p$K_a$ [207,216,217]. It was suggested that the introduced loops may not pack as well against the AMI scaffold as the native sequence [216]. The non-native loops thus may retain some flexibility that would lead to a favourable entropic term for the formation of the Cu(I)–N(His) bond resulting in the decreased p$K_a$ value. Shortening the C-terminal loop, by introducing the AMI sequence into PAZ [218] and PC [219], leads to an increased p$K_a$ from 4.9 to 6.7 (identical to that found in AMI). This demonstrates that in these three cupredoxins (AMI, PAZ and PC) the length of the loop between the His and Met ligands (the loop length between the Cys and the His ligands is identical) does regulate the p$K_a$ of the acid transition, perhaps by altering the strength of the Cu(I)–N(His) bond [208]. An entropic term originating from a lowered flexibility, as a result of the introduction of a shorter loop into PAZ and PC, may be a contributing factor.

The C-terminal His ligand does not protonate in the accessible pH range in the PC from *D. crassirhizoma* (this is the only PC which does not exhibit this phenomenon) [143,183,184]. The active site loop of this protein is identical in length to those of other PCs, but the sequence is slightly different. However, only five of the nine residues in this region are conserved among the PCs. It has been suggested that the hydrogen bond between the backbone NH of the residue two after the Cys ligand and the coordinating

| Protein | Sequence of the C-terminal ligand-containing loop | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AMI | **Cys** | Thr | | Pro | | **His** | Pro | | | Phe | **Met** |
| PC | **Cys** | Ser | | Pro | | **His** | Gln | Gly | Ala | Gly | **Met** |
| PAZ | **Cys** | Thr | | Pro | | **His** | Tyr | Gly | Met | Gly | **Met** |
| AZ | **Cys** | Thr | Phe | Pro | Gly | **His** | Ser | | Ala | Leu | **Met** |
| RST | **Cys** | Gln | Ile | Pro | Gly | **His** | Ala | Ala | Thr | Gly | **Met** |

Fig. 7. Alignment of the amino acid sequences of the C-terminal ligand-containing loops of certain cupredoxins with the coordinating residues in bold.

thiolate sulfur, absent in AMI, PAZ and PC, provides additional stabilisation of the active site which may prevent His ligand protonation in AZ for example [66]. Residue 86 is a Pro in *D. crassirhizoma* PC and thus, like all other PCs, this second hydrogen bond to the Cys ligand is missing and thus is not the cause of the absence of the acid transition. This hydrogen bond does influence the acid transition as the mutation of the corresponding Pro (residue 94) to a Phe in AMI, which introduces this second hydrogen bond [220], results in a large decrease in the p$K_a$ of the C-terminal His95 ligand from ∼7 to below 5 (the Pro94Ala mutation results in a decrease in the p$K_a$ of His95 to 6.3) [221]. Furthermore, the introduction of the AMI loop into AZ results in the C-terminal His ligand protonating, albeit with a p$K_a$ of 5.5 [219] (this AZAMI variant not only has a shorter loop than AZ but it is also missing the second hydrogen bond to the thiolate sulfur of the Cys ligand). The presence of this hydrogen bond will probably influence the flexibility of the C-terminal loop and thus an entropic effect may be prevalent. However, this hydrogen bond is present [75] in the phytocyanins which exhibit the acid transition where further loop stabilisation may result from a disulfide bridge close to the active site (see Section 7).

The inability of His87 to protonate in *D. crassirhizoma* PC has been attributed [143] to the π–π interaction between its imidazole moiety and the phenyl ring of Phe12 (see Fig. 8), which could stabilise the bound form of the His [183,184] (a Leu is usually found in this position in PCs). This is supported by the observed decrease in the p$K_a$ for His81 in the Cu(I) PAZ variant in which a π–π interaction with its coordinated imidazole has been introduced by making the Met16Phe mutation (see Fig. 8) [213]. However, the Leu12Phe mutation in spinach PC results in the p$K_a$ of His87 increasing from 4.9 in the Cu(I) wild type (WT) protein to 5.7 [143,213], and the precise arrangement of the imidazole and phenyl rings is probably an important factor. The packing of the His117 ligand between the bulky side-chains of Met13 and Phe114 has been suggested as a reason for the low p$K_a$ of this residue in AZ [222] and the Phe114Ala mutation results in a 50 mV increase in the $E_m$ (at neutral pH) and an ∼0.4 Å increase in the Cu(II)–N(His117) bond length [223]. Interestingly structural studies have indicated that the influence of the Pro94Phe and Pro94Ala mutations on the p$K_a$ of His95 in AMI is related to the introduction of steric hindrance to the rotation of the imidazole ring [220]. However, in the structure of the Pro94Phe variant the packing of the AMI molecules prevents the movement of His95.

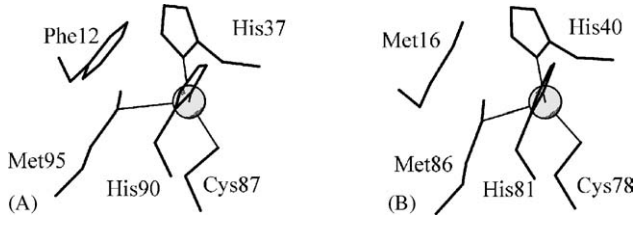



Fig. 8. Active site structure of (A) Cu(II) PC from *D. crassirhizoma* [183] (PDB accession code 1KDJ) and (B) Cu(II) PAZ from *A. cycloclastes* [60] (PDB accession code 1BQK). In both structures the four coordinating residues are shown along with the side chain of the amino acid that interacts with the imidazole ring of the C-terminal His ligand (Phe12 in *D. crassirhizoma* PC and Met 16 in PAZ). The copper ion is shown as a grey sphere in both structures.

The solvent accessibility of the active sites of cupredoxins has also been suggested as influencing the acid transition [210,211,224] and the close proximity of His87 in *D. crassirhizoma* PC to Phe12 could help to protect the His from solvent. There thus appear to be a number of factors in the cupredoxins which may be important in controlling the p$K_a$ value of the C-terminal His ligand, and further studies are needed to assess the relative importance of all of the structural features discussed. One approach which shows great promise in this respect is the use of electrochemistry at varying pH and temperature [208]. Studies carried out to date with this technique indicate that the thermodynamic driving force for the acid transition is enthalpic for PCs and entropic for a number of phytocyanins. AMI seems to be a little unusual in that both enthalpic and entropic terms favour the transition which has been assigned to undefined active site peculiarities [208].

## 6. The reactivity and interactions of cupredoxins

### 6.1. Physiological partners

Cupredoxins function as ET shuttles and therefore need to interact with a partner, pick up an electron and deliver it to another protein. Efficient ET and the ability to form a reactive complex with the appropriate partner are therefore essential. Interactions between ET partners are usually transient with electrostatics enhancing association and pre-orienting partners, whereas hydrophobic interactions (which are effective over shorter distances) ensure reactive encounter complex formation [225,226]. Physiological partners are only known with certainty for PC, AMI, PAZ and RST. PC functions as an electron transporter between the membrane-bound cytochrome $b_6f$ complex (cyt $b_6f$) and $P700^+$ of Photosystem I (PSI) in photosynthetic organisms [31,226]. AMI accepts electrons from methylamine dehydrogenase (MADH) which are then delivered, probably via a c-type cytochrome, to a cytochrome *c* oxidase in methylotrophic bacteria [27,227]. PAZ reduces NiR, although its physiological donor is not known [35], and RST is thought to shuttle electrons between a high molecular weight cytochrome *c* and cytochrome $c_4$ [39]. Structural information is available for the complex formed between AMI and MADH [25] and also PC with the soluble domain of cyt *f* (from both plants [72] and a cyanobacterium [73]), some preliminary data has been reported for the complex between PAZ and NiR [74] and the interaction between RST and cytochrome $c_4$ has been modeled [228]. In all cases the hydrophobic patch of the cupredoxin associates close to the redox centre of the partner and ET is thought to occur

via the exposed His ligand. Acidic and basic residues on the cupredoxins are essential for these interactions and mutagenesis studies have highlighted the importance of both charged [229–235] and hydrophobic [236–238] patches. By far the best studied of these complexes is that between PC and cyt *f* and thus I will focus on this system here.

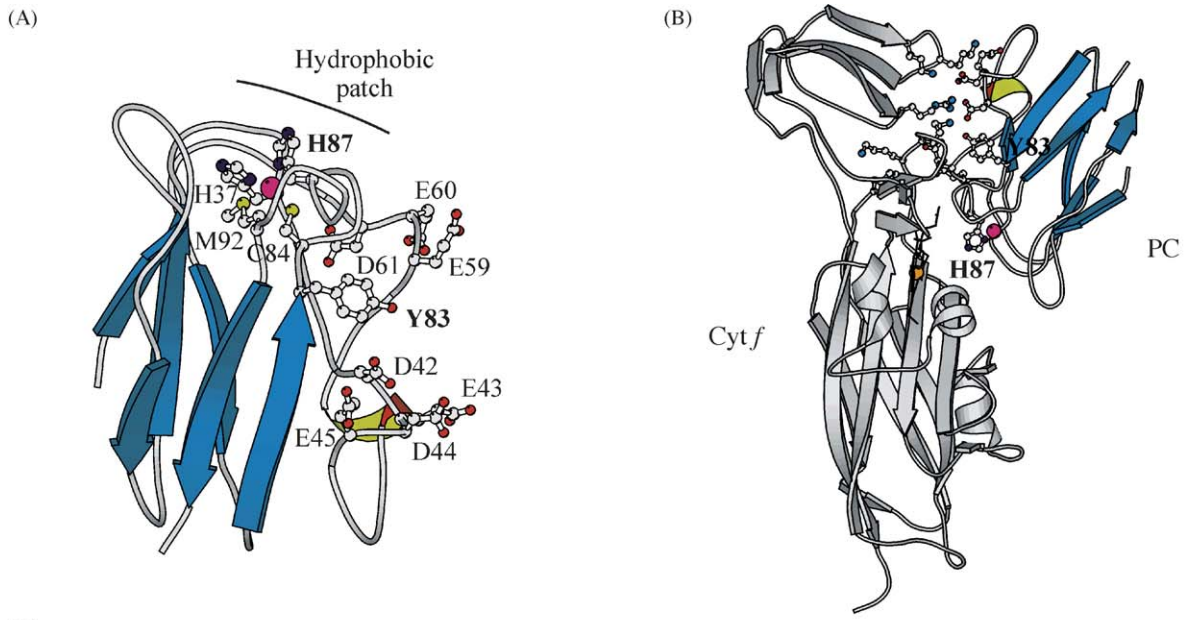
The solution structure of the complex formed by PC and cyt *f* from higher plant sources (spinach PC and turnip cyt *f*), which was determined from NMR studies utilising diamagnetic chemical shift changes and intermolecular pseudocontact shifts, is shown in Fig. 9B [72]. The patch of acidic residues on the surface of the higher plant PC around the conserved Tyr83 residue (see Fig. 9A) interact with a number of basic residues on cyt *f*. The His87 ligand of PC (see Fig. 9A) approaches the heme of cyt *f* providing an efficient ET pathway. The surface properties of PC vary depending on the type of organism from which the protein originates (see Fig. 9C and D). In green algae (for example *U. pertusa*) and the plant PC from parsley [128,142] the acidic patch is more diffuse [186] whilst in the PC from *D. crassirhizoma* very few acidic residues are found around Tyr83, and an arc of aspartates and glutamates are located on the periphery of the hydrophobic patch [183]. Cyanobacterial PCs (such as those from *Synechococcus* [187] and *A. variabilis* [130]) possess very few charged surface residues and have a more extensive hydrophobic patch. Consequently, the structure of the complex between PC and cyt *f* from the cyanobacterium *Phormidium laminosum* is dominated by hydrophobic interactions [73]. Recent studies on the interaction between PC and cyt *f* from *A. variabilis* (referred to as *Nostoc* sp. PCC7119) indicate that in this cyanobacterium electrostatics seem to be more important [240].

To investigate the effect of the altered acidic patch of parsley PC (and also the green algal proteins) on its interactions the structure of the complex with cyt *f* has been investigated by NMR spectroscopy and protein docking simulations [241]. Mapping the residues of Cu(I) parsley PC whose $^{1}$H NMR resonances are most affected by the addition of Fe(II) cyt *f* onto a space-filling model of the structure [242] reveals a distinct binding patch involving the non-polar residues which surround His87 (Fig. 10A). Remote from the hydrophobic patch are several affected amino acids, diffusely distributed about the acidic patch of the molecule. A comparison of the chemical-shift maps of parsley and spinach PC [242] in the presence of cyt *f* highlights significant variation in the binding site of these homologous proteins (Fig. 10). This suggests that parsley PC adopts a different orientation in the complex with cyt *f* which is consistent with severe steric clashes observed when parsley PC is modeled [241] into the structure of the plant PC–cyt *f* complex [72]. A docking orientation for parsley PC with cyt *f* has a rmsd of 8.8 Å from the NMR structure of the spinach PC/cyt *f* complex and a copper to iron distance of 13.4 Å conducive with fast ET (see Fig. 11) [241]. Thus, the alterations in the acidic patch of parsley PC compared to the spinach protein results in them (and probably also the PCs from green algal sources) having different orientations in their complexes with cyt *f* [241].

To assess the importance of the extent and location of the acidic patch for physiological function the ionic strength dependence of the ET reaction between a variety of PCs and a plant cyt *f* have been studied (see Fig. 12) [241,243,244]. An acidic patch on the surface of eukaryotic PCs is essential for this interaction because when this region is absent, as in the *Synechococcus* PC, the association with cyt *f* is dramatically diminished at low ionic strength (see Fig. 12) [243,244]. Small changes in the acidic patch (as in the parsley and green algal proteins) have a minimal effect on the interaction with cyt *f* (see Fig. 12) [241,244]. Thus, although the structures of the complexes of these PCs with cyt *f* are different from that of the spinach protein their acidic patches are still as efficient at promoting association. Remarkably, the relocation of the acidic patch (as in the D. *crassirhizoma* PC) has very little influence on the $k_2$ value for the reaction with cyt *f* at low *I* (see Fig. 12) [244]. Docking studies with cyt *f* have demonstrated that the relocation of the acidic patch leads to a different orientation of *D. crassirhizoma* PC which results in the arc of acidic residues interacting with those basic residues on cyt *f* which are used in the complex with spinach PC. Therefore, the relocated acidic patch on the *D. crassirhizoma* PC is still able to effectively enhance association with cyt *f* giving a complex conducive to fast ET. Thus, for a PC to be able to interact efficiently with a eukaryotic cyt *f* in vitro it has to have a hydrophobic patch close to the active site which has acidic residues at its periphery. These conclusions are consistent with the fact that a number of soluble ET proteins are able to interact with various partners [226,245,246]. Thus, the need to maintain the efficient flow of electrons along an ET chain seems to require the protein–protein interactions involved to be pseudo-specific [246].

### 6.2. Electron self-exchange reactivity

The electron self-exchange (ESE) reaction is an intrinsic property of all redox systems [247] and is an extremely useful reaction to study because the structure of only one protein needs to be considered when interpreting the rate constants ($k_{ese}$). Furthermore, the reaction has no driving force and thus provides a relative measure of the ET capabilities of the different members of a family of redox proteins. The ESE reactivity of a number of redox metalloproteins has been studied [138,142–144,178,248–271]. The available $k_{ese}$ values for cupredoxins are listed in Table 5 and range from $\sim 10^3$ to $10^6$ $M^{-1}$ $s^{-1}$ at moderate to low *I*. The presence of an acidic patch (in plant and green algal PCs) [178,271], or basic residues (as in PAZ) [144,180], close to the hydrophobic patch disfavours ESE at low *I*. ESE is thought to involve two cupredoxins associating via their hydrophobic patches, with an ET route involving the exposed His ligands [258,264,272,273]. The ET reactivity of AZ dimers covalently joined via their hydrophobic patches has been studied [272]. In the presence of a short crosslink the




(C)

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                                  20                                  40                                  60
S.pratensis   ..AEVLLGSS DGGLAFVPSD LSIASGEKIT FKNNAGFPHN DLFDEDE.VP ..AGVDVTKI
Spinach       ..VEVLLGGG DGSLAFLPGD FSVASGEEIV FKNNAGFPHN VVFDEDE.IP ..SGVDAAKI
Parsley       ..AEVKLGSD DGGLVFSPSS FTVAAGEKIT FKNNAGFPHN IVFDEDE VP ..AGVNAEKI
U.pertusa     .AQIVKLGGD DGSLAFVPSK ISVAAGEAIE FVNNAGFPHN IVFDEDA.VP ..AGVDADAI
D.crass       ..AKVEVGDE VGNFKFYPDS ITVSAGEAVE FTLVGETGHN IVFDIPAGAP GTVASELKAA
A.variabilis ETYTVKLGSD KGLLVFEPAK LTIKPGDTVE FLNNKVPPHN VVFDAAL.NP ..AKSADLAK
                   *  *    *   * *       *      *       **    **     *

                                  80                                 100
S.pratensis   SMPEEDLLNA PGEEYSVTL. ..TEKGTYKF YCAPHAGAGM VGKVTVN.
Spinach       SMSEEDLLNA PGETYKVTL. ..TEKGTYKF YCSPHQGAGM VGKVTVN.
Parsley       SQPE..YLNG AGETYEVTL  ..TEKGTYKF YCEPHAGAGM KGEVTVN
U.pertusa     SY..DDYLNS KGETVVRKL. ..STPGVYGV YCEPHAGAGM KMTITVQ.
D.crass       SMDENDLLSE DEPSFKAKV. ..STPGTYTF YCTPHKSANM KGTLTVK.
A.variabilis SLSHKQLLMS PGQSTSTTFP ADAPAGEYTF YCEPHRGAGM VGKITVAG
             *      *                      * *   ** **   * *     **
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(D)

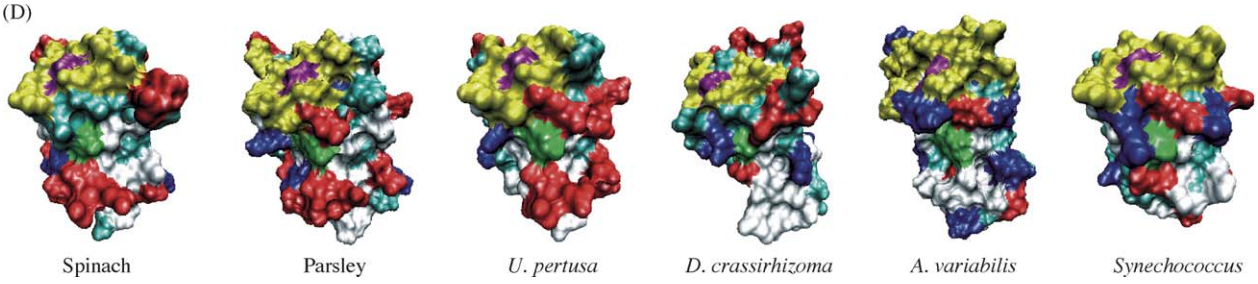



Fig. 9. (A) The structure of spinach PC (PDB entry 1AG6) [185]. The coordinating side-chains and the acidic patch surrounding the conserved Tyr83 residue are shown in ball-and-stick representation, the copper is shown as a magenta sphere and the position of the surface hydrophobic patch is indicated. The acidic patch is made up of the upper (E59, E60 and D61) and lower (D42, E43, D44 and E45) regions. (B) The structure of the complex of spinach PC with turnip cyt *f* (PDB entry 2PCF) [72]. The His87 ligand of PC is indicated as is Tyr83 and the surrounding acidic patch. A number of basic residues on the surface of cyt *f* are also included. The iron of cyt *f* and the copper of PC are shown as orange and magenta spheres, respectively. (C) Sequence alignment of the PCs from *S. pratensis*, spinach, parsley, *U. pertusa, D. crassirhizoma* and *A. variabilis* obtained using DbClustal [239]. Fully conserved residues, including the copper ligands are marked with an asterix. Charged residues which contribute to the acidic patches (plant and green algal PCs) or which are located near the hydrophobic patch are in red (acidic) and blue (basic) and Tyr83 is green. (D) The surface properties of PCs from spinach (PDB entry 1AG6), parsley (PDB entry 1PLA) [128], *U. pertusa* (PDB entry 1IUZ) [186], *D. crassirhizoma* (PDB entry 1KDJ) [183] *A. variabilis* (PDB entry 1NIN) [130] and *Synechococcus* (PDB entry 1BXU) [187] in which the exposed imidazole ring of His87 is shown in purple and the surrounding hydrophobic patch is yellow. The acidic and basic residues are red and dark blue, respectively, polar residues are cyan and Tyr83 is green.

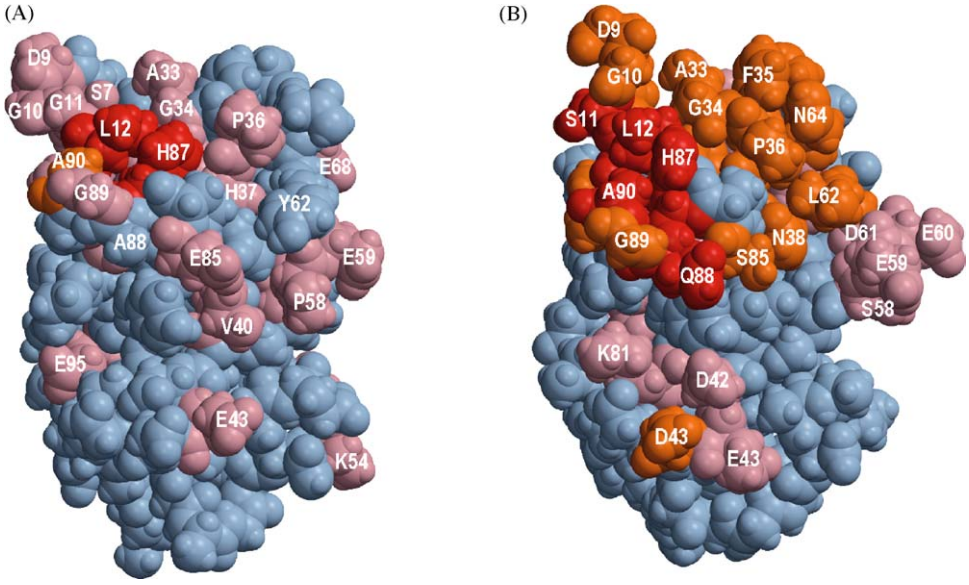



Fig. 10. Chemical-shift maps of parsley (A) and spinach (B) PC in the presence of 0.3 M equivalents of turnip cyt *f* [241]. The map for the spinach protein is reproduced from Edjebäck et al. [242]. Protein structures are represented as space-filling models and the residues are coloured according to the size of the largest chemical shift observed for any proton resonance in that residue. Blue, insignificant (<0.02 or 0.03 ppm in parsley and spinach PC, respectively); pink, 0.02–0.05 ppm or 0.03–0.05 ppm in parsley and spinach PCs, respectively; orange, 0.05–0.10 ppm; red, ≥0.10 ppm. Reproduced from [241] ©The Biochemical Society.

monomers rotate away from each other leaving the hydrophobic patches exposed and allowing ESE with other dimers. A longer linker gives a structure in which the hydrophobic patches are part of the dimer interface (analogous to the non-covalent dimer arrangement seen in the crystal structure of native AZ [62]) and fast inter-molecular ET is observed.

The $k_{ese}$ values of PCs are dependent on the source (surface properties) of the protein and cover the range exhibited

Table 5
Electron self exchange rate constants ($k_{ese}$) of cupredoxins[a,b]

| Protein | Charge | $k_{ese}$ ($M^{-1}\,s^{-1}$) | Conditions | Reference |
|---|---|---|---|---|
| AZs | | | | |
| *P. aeruginosa* | 0 | $9.6 \times 10^5$ | pH 4.5, $I$ = 20 mM (25 °C) | [253] |
| | 0 | $7.0 \times 10^5$ | pH 9.0, $I$ = 60 mM (25 °C) | [253] |
| *A. denitrificans* | +2 | $4.0 \times 10^5$ | pH 6.7, $I$ = 38 mM (24 °C) | [254] |
| Asn42Cys disulfide homodimer[c] | | $4.2 \times 10^5$ | pH 8.5, $I$ = 74 mM (40 °C) | [272] |
| Asn42Cys linked homodimer[d] | | $\geq 5 \times 104\,s^{-1}$ (intramolecular $k_{et}$) | pH 8.5, $I$ = 74 mM (40°C) | [272] |
| PCs | | | | |
| Spinach | −9 | $2.5 \times 10^3$ | pH 8.0, $I$ = 100 mM (25 °C) | [178] |
| Parsley | −7 | $5.0 \times 10^4$ | pH 7.5, $I$ = 100 mM (25 °C) | [142] |
| *U. pertusa* | −7 | $3.5 \times 10^4$ | pH 8.0, $I$ = 100 mM (25 °C) | [178] |
| *D. crassirhizoma* | −6 | $3.4 \times 10^3$ | pH 7.9, $I$ = 100 mM (25 °C) | [143] |
| *A. variabilis* | +2 | $2.4 \times 10^5$ | pH 6.2, $I$ = 100 mM (25 °C) | [178] |
| AMI | −3 | $1.3 \times 10^5$ | pH 8.6, $I$ = 50 mM (25 °C) | [138] |
| PAZ | +2 | $3.7 \times 10^3$ | pH 7.6, $I$ = 100 mM (25 °C) | [144] |
| | | $1.1 \times 10^3$ | pH 6.0, $I$ = 100 mM (25 °C) | [144] |
| | | $1.7 \times 10^4$ | pH 10.9, $I$ = 100 mM (25 °C) | [180] |
| RST | +5 | $1.7 \times 10^4$ | pH 2.0, $I$ = 100 mM (25 °C) | [265] |
| | | $1.0 \times 10^4$ | pH 5.7, $I$ = 100 mM (25 °C) | [265] |
| STCs | | | | |
| *R. vernicifera* | +8 | $1.2 \times 10^5$ | pH 7.0, $I$ = 225 mM (20 °C) | [248] |
| Horseradish (UMC) | −2 | $1.8 \times 10^4$ | pH 7.5, $I$ = 100 mM (40 °C) | [269] |
| CBP (a PLN) | +6 | $6.0 \times 10^5$ | pH 8.0, $I$ = 100 mM (25 °C) | [270] |

[a] All determined by NMR spectroscopy except in the case of *R. vernicifera* STC for which EPR was used.
[b] All measured, except for *R. vernicifera* STC, in >99% $D_2O$ with pH meter readings uncorrected for the deuterium isotope effect (except for AMI).
[c] AZ homodimers from *P. aeruginosa* linked via a disulfide bridge formed using a Cys residue introduced at the edge of the hydrophobic patch (at position 42).
[d] AZ homodimers from *P. aeruginosa* connected via a bis-maleimidomethylether linker using a Cys residue introduced at the edge of the hydrophobic patch (at position 42).

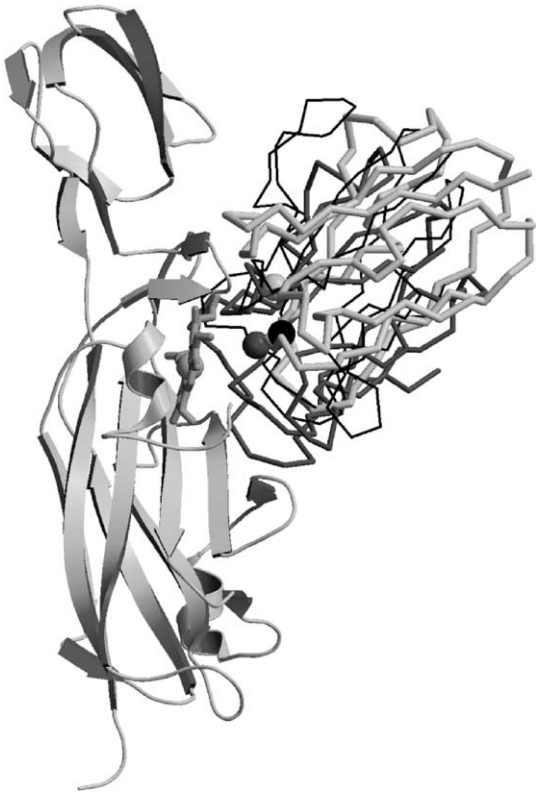


Fig. 11. Superimposition of the results from docking simulations with the NMR structure of the PC–cyt *f* complex [72]. Cyt *f* is represented as a ribbon diagram, with the α-helix in front of the haem removed for clarity. PC structures are represented as $C^{\alpha}$ backbone traces. Spinach PC from the NMR structure of the complex (thin trace, black), the top-ranked orientation for spinach PC (medium trace, grey line) and the third-ranked docking orientation for parsley PC (heavy trace, light grey) are shown. Reproduced from [241] ©The Biochemical Society.

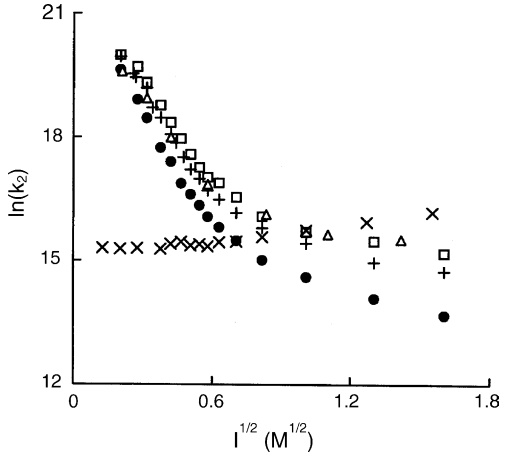



Fig. 12. The ionic strength dependence of ln of the rate constant ($k_2$) for the oxidation of turnip cyt *f* by spinach (+), parsley (△) *U. pertusa* (□), *D. crassirhizoma* (●) and *Synechococcus* (×) PCs [244]. The rate constant $k_2$ has been defined as $k_2 = k_{on}k_f/(k_{off} + k_f)$, where $k_{on}$ is the rate of association of the two proteins, $k_{off}$ is the rate of dissociation before ET and $k_f$ is related to $k_{et}$ [225]. It has been demonstrated that for the reaction between PC and cyt *f*, $k_f \gg k_{off}$ and thus $k_2 = k_{on}$.

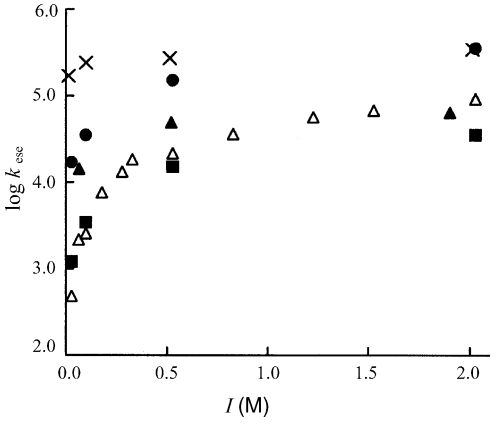
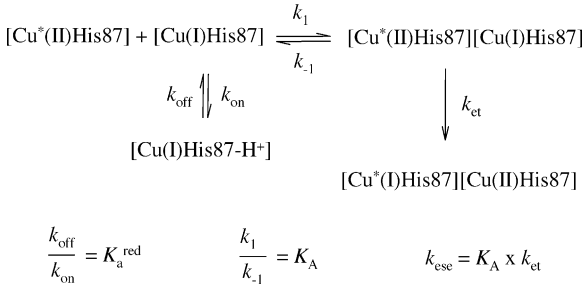



Fig. 13. Ionic strength dependence (25 °C) of $\log k_{ese}$ for spinach (△), *U. pertusa* (●), *D. crassirhizoma* (■) and *A. variabilis* (×) PCs [271]. The ionic strength was adjusted by the addition of NaCl to 10 mM phosphate buffer (99.9% $D_2O$) $pH^*$ 8.0 for spinach, *U. pertusa* and *D. crassirhizoma* PCs and at $pH^*$ 6.2 for *A. variabilis* PC. For the $k_{ese}$ determination of *U. pertusa* PC at $I = 0.10$ M, 35 mM phosphate buffer $pH^*$ 8.0 was used and 73 mM phosphate buffer ($pH^*$ 6.2) was utilised for the *A. variabilis* PC measurement at $I = 0.10$ M. The $k_{ese}$ value for *D. crassirhizoma* PC at $I = 0.10$ M was taken from [143] (36 mM phosphate buffer at $pH^*$ 7.9). Also included is the ionic strength dependence (25 °C) of $\log k_{ese}$ for spinach PC in 10 mM phosphate buffer (99.9% $D_2O$) at $pH^*$ 8.0 with added $MgCl_2$ (▲).

by cupredoxins [142,143,178,249,261,271]. The influence of ionic strength on $k_{ese}$ of various PCs are shown in Fig. 13 [271]. The acidic patches of spinach, *D. crassirhizoma* and *U. pertusa* PCs result in electrostatic repulsion which hinders protein association and leads to a small $k_{ese}$ value at low *I*. Screening of the charged patches (by the addition of NaCl) at elevated *I* leads to 190-, 29- and 21-fold increases in $k_{ese}$ for the spinach, *D. crassirhizoma* and *U. pertusa* proteins, respectively. The largest accelerating effect of *I* on $k_{ese}$ in spinach PC (and the smallest $k_{ese}$ at low *I*) correlates with it being the most acidic of the PCs and thus electrostatic repulsion has the most significant effect on homodimer formation. In contrast to the other PCs, the $k_{ese}$ for the *A. variabilis* protein is large at low *I* and exhibits very little dependence on ionic strength (see Fig. 13). The $k_{ese}$ values at high *I* for the PCs are consistent with those observed for other redox metalloproteins under similar conditions [178,255,257,262,267].

The ionic strength dependence of the $k_{ese}$ of the PCs has been analyzed using van Leeuwen theory, which considers monopole–monopole, monopole–dipole and dipole–dipole interactions involved in protein–protein association [255,257,268,271,274] to give $D'_{ox}$, $D'_{red}$ and $k_{inf}$ (rate constant at infinite *I*) values of −141, −159 D and $1.5 \times 10^5\ M^{-1}\ s^{-1}$, respectively. The $D'_{ox}$ and $D'_{red}$ values of −141 and −159 D, respectively for spinach PC represent the components of the dipole moment through the site on the protein (oxidised and reduced PC) via which ESE occurs. The components of the dipole moments through the hydrophobic patch range from −160 to −40 for spinach PC [271]. Thus, the values obtained from the van Leeuwen fits are consistent with homodimer formation via the hydrophobic patch which




Scheme 1.

surrounds the exposed His ligand [258,264,272]. Fitting of the data for *U. pertusa*, *D. crassirhizoma* and *A. variabilis* PCs, respectively, gives $k_{inf}$ values of $4.9 \times 10^5\ M^{-1}\ s^{-1}$, $7.3 \times 10^4\ M^{-1}\ s^{-1}$ and $3.3 \times 10^5\ M^{-1}\ s^{-1}$ [271]. Hydrophobic interactions are favoured at high $I$ and the small $k_{inf}$ for *D. crassirhizoma* PC can be partly explained by the less hydrophobic binding site in this protein (see Fig. 9D). ESE is an activation controlled reaction and thus $k_{ese} = K_A k_{et}$, where $K_A$ is the association constant for encounter complex formation and $k_{et}$ is the rate constant for ET (see Scheme 1) [275]. Differences in $k_{inf}$ therefore could be due to variations in $k_{et}$ which is influenced by the electronic coupling between the donor and the acceptor (distance for ET) and the reorganisation energy for ET. Thus, the relatively small $k_{inf}$ for spinach PC and the lower than anticipated $k_{inf}$ for the *A. variabilis* protein (considering that it possesses a large hydrophobic patch) could both be due to diminished $k_{et}$ values (vide infra).

Protein modeling based on the crystallographic dimer of *Silene pratensis* PC [276] (the amino acid sequence of this PC is included in Fig. 9C) and docking simulations of the various PCs have been used to obtain representative structures of the transient homodimers involved in ESE [271]. The molecules associate via their hydrophobic patches and thus these models are consistent with the fits of the ESE data to van Leeuwen theory and agree with the proposed structure of the AZ ESE homodimer [258,264,272,273]. The two PCs assume an orientation in which the acidic patches are located on opposite faces of the homodimer, thus minimising electrostatic interactions. The Coulombic repulsion between the monomers in the docked complexes follows the order: spinach > *D. crassirhizoma* > *U. pertusa* > *A. variabilis*, which is in good agreement with the observed $k_{ese}$ values at low $I$ and matches the relative accelerating effect of increasing $I$ on $k_{ese}$ (see Fig. 13). These complexes therefore provide structural models for explaining the influence of electrostatics on ESE [271]. A more polar dimer interface for *D. crassirhizoma* PC is consistent with the small $k_{inf}$ for this protein. The *A. variabilis* PC dimer has a large number of hydrophobic residues in the interface which along with the relatively small $k_{inf}$ value probably indicates that $k_{et}$ is smaller in this protein. This is consistent with the Cu–Cu distance being largest in the *A. variabilis* PC docked complex.

Increasing the concentration of $MgCl_2$ has a much more pronounced influence than NaCl on the $k_{ese}$ of spinach PC (see Fig. 13) indicating specific interactions between $Mg^{2+}$ ions and the protein [249,271]. The $Mg^{2+}$ ion is present in the thylakoid lumen at approximately mM concentrations [277] and has been implicated in catalysis of the oxidation of PC by PSI [278]. The observed accelerating influence of similar concentrations of $Mg^{2+}$ ions on the ESE of PC along with the high protein concentration (>20 mg/mL [279]) within the thylakoid lumen (PC is one of the most abundant proteins in the thylakoid [279,280] and exists as a pool of both oxidised and reduced forms [50]) indicate that this reaction may occur physiologically, facilitating ET between the membrane bound cyt $b_6f$ complex and PSI.

The temperature dependence of the ESE reactivity of spinach PC yields $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values of $44\ kJ\ mol^{-1}$ and $-16\ J\ mol^{-1}\ K^{-1}$, respectively [271]. The large $\Delta H^{\ddagger}$ for spinach PC is consistent with the electrostatic repulsion which has to be overcome for homodimer formation. The fact that this reaction is also disfavoured on the grounds of activation entropy suggests that displacement of water molecules from the protein interface is insufficient to counterbalance the loss of translational and rotation freedom upon protein–protein association. Thus, the experimental data provides evidence of poor packing in the interface. This is corroborated by the interfaces in the homodimers which have low complementarity and high planarity, features which are essential to the nature of the associations involved in ESE and other transient protein interactions [226,281–283].

The influence of pH on the $k_{ese}$ of PCs has been studied to assess the role of the protonation of the His87 ligand on ET reactivity [142,178]. The $k_{ese}$ values of *U. pertusa* and parsley PCs are almost independent of $pH^*$ (at $I = 0.10$ M), even at values where His87 is protonated and thus the Cu(I) molecules will possess a three-coordinate active site (see Fig. 14). This form of PC is known to be redox-inactive [206,214,215] and a smaller ESE rate constant would be expected (see Scheme 1 where [Cu(I)His87-$H^+$] is the form of reduced PC in which His87 is protonated and is the redox inactive species). This absence of any pH-dependence of the ESE reactivity can be attributed to compensation effects caused by the presence of acidic patches in these PCs. At lower pH the acidic residues start to protonate, thus, neutralising the charge in this region of the protein leading to a larger $K_A$ (see Scheme 1). This is confirmed by the decrease in the $k_{ese}$ of *A. variabilis* PC at lower pH (see Fig. 14) and by the increased $k_{ese}$ of *D. crassirhizoma* PC at lower $pH^*$ (due to the absence of His87 protonation [143]). Furthermore, at high ionic strength ($I \sim 0.50$ M, see Fig. 14) the $k_{ese}$ of *U. pertusa* PC decreases at low pH. Thus, as the pH is lowered more of the reduced protein exists as [Cu(I)His87-$H^+$] (see Scheme 1) which is redox inactive and ESE reactivity is diminished. It would perhaps be expected that ESE would be more dramatically affected at low pH. The fact that the PCs are still reasonably competent at ESE under these conditions suggests that the process has now become gated (by

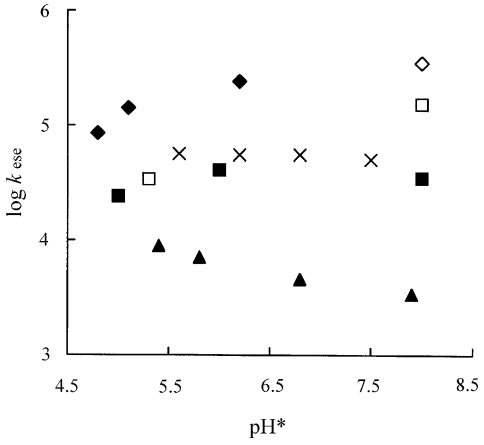



Fig. 14. Dependence (25 °C) on pH* of log $k_{ese}$ of *U. pertusa* PC in phosphate buffer at $I = 0.10$ M (■), *U. pertusa* PC in 10 mM phosphate buffer plus 0.50 M NaCl (□), *A. variabilis* PC in phosphate buffer at $I = 0.10$ M (♦), parsley PC in phosphate buffer at $I = 0.10$ M (×) and *D. crassirhizoma* PC in phosphate buffer at $I = 0.10$ M (▲) [178]. Also included is data for *Synechococcus* PC in phosphate buffer at $I = 0.10$ M (◊).

the activation barrier for the transition from three-coordinate [Cu(I)His87-$H^+$] to four coordinate [Cu(II)His87]).

The acid transition of PC and other reduced cupredoxins may play an important physiological function. For example, it is thought that His87 protonation could provide a metabolic feedback mechanism whereby the drop in pH resulting from a high rate of photosynthesis would result in a decrease in ET activity [77,284]. The fact that the reduction of $P700^+$ by PC is hardly affected at low pH [285] therefore seems a little puzzling. It may be that the approach of a non-polar surface of $P700^+$ to the hydrophobic patch of [Cu(I)His87-$H^+$] destabilises the positive charge on His87 and thus [Cu(I)His87], the redox active species, would be rapidly reformed (see Scheme 1). Therefore, His87 protonation may provide a way of ensuring specificity of photosynthetic ET as the reaction with other oxidants would be expected to be much more significantly gated. A similar, potentially physiologically relevant, pH-dependent transition ($pK_a$ 4.8) has been characterised in the $Cu_A$ loop variant (see Section 8.2) of AZ [286] and the C-terminal His ligand has been identified as the site of protonation. As the corresponding His is in the major ET route from $Cu_A$ to heme *a* in cytochrome *c* oxidase, it has been suggested that this ligand could play a regulatory role in proton-coupled ET.

Studies investigating the features of cupredoxins (mainly PC) important for their interactions have been reviewed. The presence of surface charged residues are important for facilitating association with physiological partners. There is some flexibility in the location of these recognition patches and in the case of the eukaryotic PCs the presence of an acidic region on the periphery of the hydrophobic patch is all that is required for efficient reactivity with cyt *f*. The presence of surface charged regions close to the hydrophobic patch hinders ESE at moderate ionic strength. For all reactions hydrophobic interactions are important and the hydrophobic patch of the cupredoxin is utilised for ET via the exposed His ligand. A number of outstanding issues have not been resolved concerning the important aspects of the surface of a cupredoxin which ensure recognition whilst maintaining rapid ET. How these features relate to the reactivity of cupredoxins in vitro also needs to be considered in more detail. On this issue it is interesting to note that electrostatics seem to be less important for the interaction between eukaryotic PC and cyt *f* in vivo [287]. The elucidation of ET partners (and their structures) for more of the members of the cupredoxin family is required. As these are identified the structures of the complexes can be studied and a more detailed understanding of the interactions involved will be achieved. For example, the recent studies on PAZ and NiR indicate that the complex formed is not transient which seems to contradict its observed reactivity [74].

## 7. The phytocyanins—a sub-family of the cupredoxins

The phytocyanins form a sub-family of the T1 copper-containing proteins which contain a single cupredoxin domain and interrogation of DNA databases suggests that all vascular seed plants will probably synthesise one or more of these proteins [50,53,70,288]. Some plants produce a large number of phytocyanins and *Arabidopsis thaliana* possesses at least 42 phytocyanin-related genes [50]. Phytocyanins have a number of unusual structural features compared to other cupredoxins including a twist in one of the β-sheets that makes up the β-barrel, a disulfide bridge close to the active site, and solvent accessibility of the imidazole rings of both His ligands [43,46,66,75]. The phytocyanins have been divided into three sub-groups [STCs, uclacyanins (UCAs) and PLNs] on the basis of domain organisation, glycosylation and the nature of the axial ligand [53]. The PLNs and UCAs usually have an axial Met ligand like most other cupredoxins whereas the STCs are identified by an axial glutamine ligand (see Figs. 2 and 15) and thus, along with fungal LACs [68,71] and ceruloplasmin [13] provide one of the very few naturally occurring T1 sites with an altered coordination environment (a couple of PLNs have been found to have either a Val or Leu in the axial position [50,53,69,70]). The STCs and UCAs possess associated carbohydrate and a putative cell wall anchoring domain [53,289] whereas the PLNs have neither of these features (see Fig. 15).

Umecyanin (UMC) is a STC isolated from horseradish roots [42] and sequence alignments indicate that its axial ligand is the side chain of Gln95 [290] (see Fig. 15). Despite the apparent conservation of the STC metal-binding ligands UMC is unusual in that it possesses a classic T1 copper site (low absorbance at ∼450 nm and an axial EPR spectrum) [175,295,296] whereas other STCs have perturbed T1 centres (see Fig. 16). To enable detailed studies of UMC an artificial coding region for its cupredoxin domain has been designed

```
UMC                  --EDYDVGG-DMEWKRPS-DPKFYI-TWATGKTFRVGDELEFDFAAGMHDVAVVT
BCB                  --EDYDVGD-DTEWTRPM-DPEFYT-SWATGKTFRVGDELEFDFAAGRHDVAVVS
R. vernicifera STC   --TVYTVGD-SAGWKVPFFGDVDYDWKWASNKTFHIGDVLVFKYDRRFHNVDKVT
Cucumber STC         QSTVHIVGD-NTGWSVPSS-PNFYS-QWAAGKTFRVGDSLQFNFPANAHNVHEME

A. thaliana UCCI     --TDHTIGGP-SGWTVGASLR-----TWAAGQTFAVGDNLVFSYPAAFHDVVEVT

CBP                  --AVYVVGG-SGGWTFN-------TESWPKGKRFRAGDILLFNYNPXMHNVVVVN


UMC                  -KDAFDNCKKENPISHMTTP-PVKIMLN-TTGPQYYICTVGDHCRVGQKLSINVV
BCB                  -EAAFENCEKEKPISHMTVP-PVKIMLN-TTGPQYFICTVGDHCRFGQKLSITVV
R. vernicifera STC   -QKNYQSCNDTTPIASYNTG-NNRINLK-TVGQKYYICGVPKHCDLGQKVHINVT
Cucumber STC         TKQSFDACNFVNSDNDVERTSPVIERLD-ELGMHYFVCTVGTHCSNGQKLSINVV

A. thaliana UCCI     -KPEFDSCQAVKPLITFANG-NSLVPLTTPGKR-YFICGMPGHCSQGMKLEVNVV

CBP                  -QGGFSTCNTPAGAKVYTSG--RDQIKLPK-GQSYFICNFPGHCQSGMKIAVNAL 96


UMC                  GAGGAGG-----GATPGA---------------------- 115
BCB                  AAGATGGATLGAGATPALGSTPSTGGTTPPTAGGTTTPSG 142
R. vernicifera STC   VRS----------------------------------- 107
Cucumber STC         AANATVSMPPPSSSPPSSVMPPP----------------- 129

A. thaliana UCCI     PTATVAPTAPLPNTVPSLNAPSPSSVLPIQPLLPLNPVPV 139
```

Fig. 15. Multiple sequence alignment of selected phytocyanins generated using Clustal-W [291]. Included are UMC from horseradish roots (a STC) [290], BCB (a STC) from *A. thaliana* [47], *R. vernicifera* STC [292] and cucumber STC (CST) [293]. Also shown are UCCI (a UCA from *A. thaliana* [53]) and CBP (the PLN from cucumber [294]). The residues known, or thought to be copper ligands are shown in bold and the Cys residues involved in the disulfide bond are underlined. Parts of the C-terminal extensions of UMC, BCB, CST and UCCI, which are thought to form cell-wall anchoring domains, are included (the cupredoxin domain of UMC probably finishes at Gly106).

and synthesised [295] and the recombinant protein is identical to the native molecule from horseradish roots (recombinant BCB, a STC from *A. thaliana* is also remarkably similar and possesses a classic T1 site [295]). Paramagnetic $^1$H NMR spectroscopy has demonstrated that UMC, CST and *R. vernicifera* STC all have the typical interaction between the copper and the $His_2Cys$ equatorial ligands observed in other structurally characterised cupredoxins (see Fig. 3 and Table 2) [70,175,179,295]. The Cys $C^{\beta}H$ resonances were indirectly observed for Cu(II) CST and their $\delta_{\beta,av}$ value (410 ppm) is smaller than those of AZ, PC and PAZ indicating a weaker Cu(II)–S(Cys) interaction (vide infra) [179]. The NMR spectra of the Cu(II) proteins do not exhibit any paramagnetically shifted resonances from the axial ligand demonstrating that the Gln does not contribute to the singly occupied molecular orbital in STCs [175,179,295].

Additional active site information has been obtained for Ni(II) UMC [175] whose NMR spectrum (see Table 3) has been assigned by observing dipolar connectivities (Fig. 17). This spectrum confirms that, as well as having a $His_2Cys$ equatorial ligand set, the active site is completed by an axial Gln ligand. The observation of the resonance from one of the $N^{\varepsilon 2}H$ protons of Gln95 indicates that this residue coordinates to Ni(II) in a monodentate fashion via the side-chain

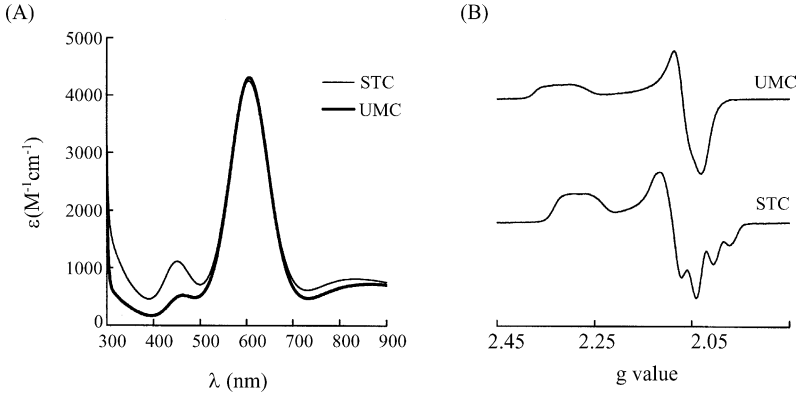



Fig. 16. Comparison of the spectroscopic properties of Cu(II) UMC and Cu(II) *R. vernicifera* stellacyanin (STC). (A) UV/vis spectra (25 °C) in 10 mM phosphate buffer pH 7.6 and (B) X-band EPR spectra (−196 °C) in 25 mM Hepes pH 7.6, 40% glycerol (bottom).

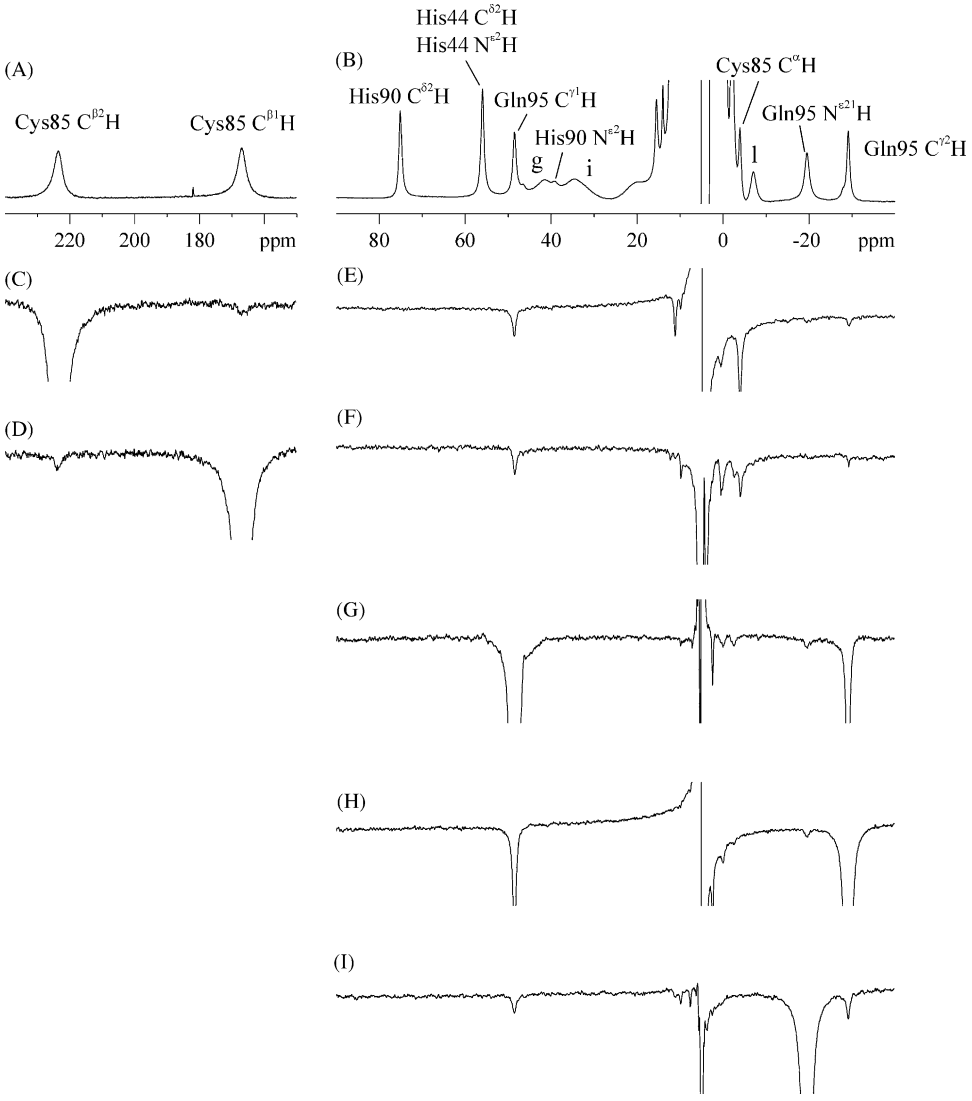



Fig. 17. Reference (A and B) and difference (C–I) $^1$H NMR spectra (300 MHz) of Ni(II) UMC corresponding to 1D NOE experiments performed in 10 mM phosphate buffer [175]. Spectra A, C and D were acquired at 30 °C with the sample in 99.9% deuterated buffer at pH* 8.0. Spectra B and E–I were measured at 10 °C with the protein in buffer in 90% $H_2O$/10% $D_2O$ at pH 6.5. In spectra C and E and D and F the two Cys $C^{\beta}H$ proton resonances were irradiated. In spectra G, H and I signals arising from the axial Gln ligand were irradiated. The main assignments that have been made are included in A and B [175] and are listed in Table 3. Peaks *g* and *i* arise from the $C^{\varepsilon 1}H$ protons of the two His ligands and signal *l* is tentatively assigned to a His ligand $C^{\beta}H$ proton.

carbonyl oxygen atom (coordination via the deprotonated side chain amide of Gln95 would result in the remaining $N^{\varepsilon 2}H$ proton being very close to the metal and being too broad to be observed). An axial Gln ligand had previously been identified from NMR studies on Co(II) and Ni(II) *R. vernicifera* STC [161,167], and the paramagnetic $^1$H NMR spectra of Cu(II) and Ni(II) UMC [175] are remarkably similar to those of other STCs [70,163,167,179]. This is surprising considering that Cu(II) UMC has distinct ultraviolet–visible (UV–vis) and EPR spectral properties (see Fig. 16). In particular the Ni(II)–O(Gln) interaction is comparable in these STCs [167,175]. Thus, the active site alterations which give rise to either classic or perturbed T1 sites in the STCs are small and do not have a significant influence on their paramagnetic NMR spectra (a similar conclusion was made from the paramagnetic NMR studies of cupredoxins with axial Met ligands, vide supra).

The crystal structures of Cu(II) and Cu(I) UMC at 1.9 and 1.8 Å, respectively, have been determined (see Fig. 18) [43]. The structure of the oxidised protein is very similar to that of CST [46], the only other available STC structure, and shows homology to those of the PLNs CBP [66] and PNC [75]. These molecules all possess the features characteristic of the phytocyanin sub-class of the cupredoxins. The largest difference between UMC (and CST) and the PLNs involves the region from Trp11 to Trp23 which forms α-helix 1 in the STCs and is absent in the PLNs. The prominent surface feature of UMC is a large acidic patch on one side of the molecule (from residues in β-strands 1 and 3) [43]. CST possesses a similarly sized acidic patch although in a

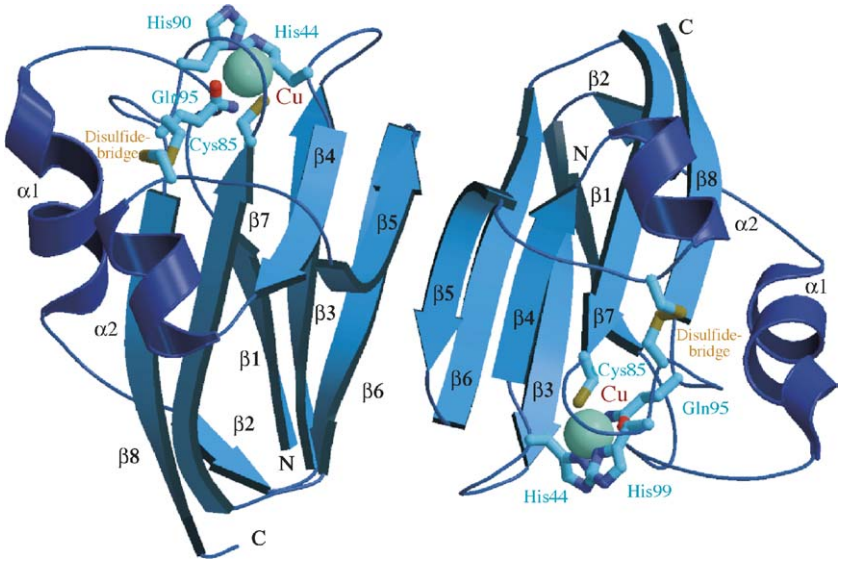



Fig. 18. The structure of UMC [43], showing the two molecules that make up the dimer arrangement in the asymmetric unit of the oxidised protein. The non-crystallographic two-fold rotation axis lies perpendicular to the plane of the paper between β-strand 5 of the two molecules. The disulfide bridge is included as are the coordinating side-chains with the copper ion shown as a cyan sphere. Reprinted from [43] © 2005 American Chemical Society.

different location (made up of amino acids from β-strands 4–6) [46]. CBP and PNC (PLNs) are basic proteins and have large positively charged surface patches [66,75]. These are found in completely different positions to the acidic surfaces on UMC and CST and are located at the opposite end of the β-sandwich to the copper site. It has been suggested that the phytocyanins may interact with small molecule redox partners [50,53,66], however the presence of charged surface patches on the phytocyanins would suggest a role in protein–protein ET (see Section 6). In the structure of UMC [43], as in those of all other phytocyanins [46,66,75], both His ligands are solvent exposed but the protein surface in this area is not exclusively hydrophobic. The lack of a clear hydrophobic patch is a feature common to the STCs and must be related to the partners with which these molecules interact. The variable features of the surface patches of the phytocyanins suggests that relatively relaxed evolutionary constraints have been imposed on the protein scaffold which has allowed a range of attributes to evolve for binding to specific partners.

The distorted tetrahedral geometry of the Cu(II) site of UMC [43], with Gln95 coordinating in a monodentate fashion via its $O^{\varepsilon 1}$ atom providing a fourth strong ligand (see Fig. 19), is as predicted from the paramagnetic NMR studies [175] and is remarkably similar to the active site of oxidised CST [46]. This arrangement is also analogous to that found at the Cu(II) site of the Met121Gln AZ mutant [297], made as a model for the active site of the STCs. It has been suggested that the

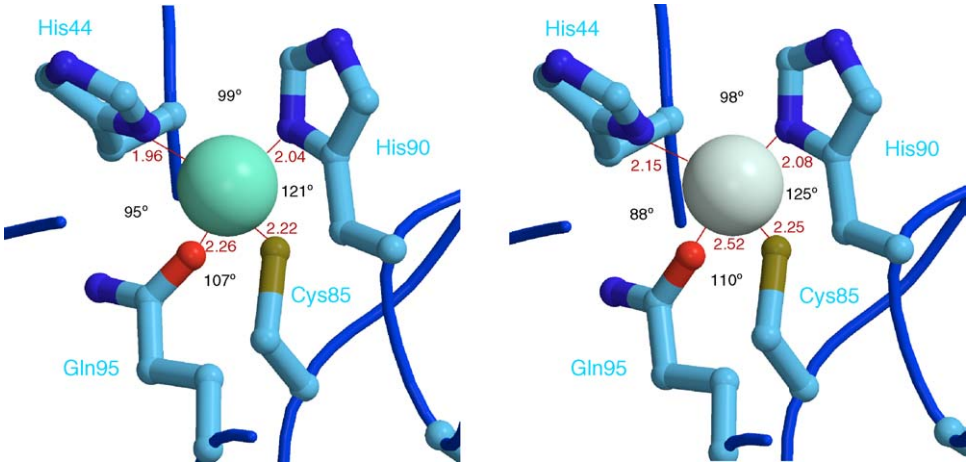



Fig. 19. The active site structures of Cu(II) (left) and Cu(I) (right) UMC with the coordinating residues shown as ball-and-stick models with the Cu–ligand distances (Å) and angles (°) included. Reprinted from [43] © 2005 American Chemical Society.

homology of the Cu(II)–$O^{\varepsilon 1}$(Gln) bond in these proteins may be due to the planar nature of the Gln sidechain [106]. The interaction of the cupric ion with the three equatorial ligands in all of these proteins is similar to that seen in cupredoxins with a weak axial Met ligand [37,41,59,60,63,65]. A possible exception to this is the Cu(II)–S(Cys) bond which appears slightly longer in the STCs. This is consistent with the results of various spectroscopic investigations [97,104,106,123,298] including paramagnetic NMR studies on Co(II) [161], Cu(II) [179] (see Table 2) and Ni(II) [167,175] (see Table 3) STCs, and has been attributed to the strong axial interaction which results in a weakening of the M(II)–S(Cys) bond. The close similarity of the active site structures of Cu(II) UMC and CST is surprising considering their spectroscopic differences (see Fig. 16). The angle between the $N_{His}CuN_{His}$ and $S_{Cys}CuO_{Gln}$ planes are 89° and 84° in Cu(II) UMC and CST, respectively, and this, along with the slightly longer Cu(II)–$O^{\varepsilon 1}$(Gln) bond in UMC (2.26 Å compared to 2.21 Å), could account for the electronic variations [97,105]. However, the source of the perturbed spectral features of sites with axial Gln ligands has been attributed to a tetrahedral, rather than tetragonal, distortion relative to the classic site of PC [106].

The largest changes at the active site of UMC upon reduction (see Fig. 19) involves the axial Gln95 ligand which twists away from the metal ion resulting in a ∼0.3 Å increase in the Cu to $O^{\varepsilon 1}$ distance [43], and a lengthening of the Cu–$N^{\delta 1}$(His44) bond by ∼0.2 Å. The geometry is slightly modified from distorted tetrahedral to more trigonal pyramidal in Cu(I) UMC. Some active site information has been reported for Cu(I) CST [299] which indicates larger changes including an increase in the Cu–$O^{\varepsilon 1}$(Gln) distance of ∼0.5 Å upon reduction. In UMC the limited alterations at the active site upon reduction are not too dissimilar from those observed at the copper centres of cupredoxins with axial Met ligands [60,63,77]. Thus UMC, and probably all STCs have active sites which are entatic [76,90,91].

A unique property of all studied phytocyanins is that they exhibit altered spectroscopic properties [for the Cu(II) protein] above pH 9 and thus exhibit an alkaline transition [45,70,163,211,295,300–304]. This effect results in a blue shift of the S(Cys) → Cu(II) LMCT bands upon increasing pH and has a p$K_a$ of ∼10. A number of suggestions have been made for the cause of the alkaline transition in the phytocyanins. A particularly attractive proposal involves a change in the coordination mode of the axial Gln ligand in the STCs from $O^{\varepsilon 1}$ at neutral pH to $N^{\varepsilon 2}H^-$ at more alkaline pH [302,305]. A conserved Lys residue adjacent to the axial ligand (see Fig. 15) has also been suggested as being responsible for this effect [70,297,304,306,307]. It has also been proposed that the phytocyanins are flexible molecules which undergo a change in secondary structure upon deprotonation of a surface residue which alters the active site structure [163,299]. However, the crystallographic studies on UMC [43] demonstrate that the active site environment of a STC is quite rigid [attempts to obtain structural information on the alkaline form of Cu(II) UMC were unsuccessful due to the instability of the crystals under these conditions]. The alkaline transition also influences the $E_m$ values of phytocyanins and thus alters their ET reactivity [34,70,211,304].

The effect of the alkaline transition on the paramagnetic NMR spectra of Cu(II) and Ni(II) UMC has been studied [175,304,308]. The main observable effect in the Cu(II) protein is a ∼1 ppm decrease in the $\delta_{obs}$ value of the Asp45 $C^{\alpha}H$ proton resonance. Similar alterations are also observed in the paramagnetic NMR spectra of Cu(II) *R. vernicifera* STC [70]. More detailed information is obtained from the spectrum of Ni(II) UMC (see Fig. 20) [175] where there is a significant decrease in the $\delta_{obs}$ values of the Cys85 $C^{\beta}H$ resonances and a lowering of the $\delta_{obs}$ between these signals at alkaline pH (analogous changes have been seen in Co(II) *R. vernicifera* STC [163]). These observations indicate a significant decrease in the Ni(II)–S(Cys) bond strength in the alkaline form and a change in the conformation of this ligand, affecting the $H^{\beta}$–$C^{\beta}$–$S^{\beta}$–Ni(II) dihedral angles. The Ni(II)–O(Gln) interaction also diminishes as a consequence of the alkaline transition, but the observation of the Gln95 $N^{\varepsilon 21}H$ proton resonance at pH 10.7 highlights that the coordination mode of the axial ligand is not altered in Ni(II) UMC at high pH (see Fig. 20). Therefore, the alkaline form of Ni(II) UMC does not involve a switch in coordination mode of the Gln95 ligand from $O^{\varepsilon 1}$ to $N^{\varepsilon 2}H^-$.

The alkaline transition has a similar influence on the spectroscopic properties of CBP which is a PLN with Met89 as the axial ligand [308]. The Gln95Met axial ligand mutation in UMC and the Met89Gln mutation in CBP have a very limited, yet similar, influence on the p$K_a$ for the alkaline transition [308]. The removal of the axial ligand in the Met89Val CBP variant results in a larger decrease in the p$K_a$, but similar spectral alterations are still observed [308]. Therefore, the axial Gln ligand is not the cause of the alkaline transition in Cu(II) STCs and alterations in the active site structures of the phytocyanins have a limited effect on this feature. The influence of increasing pH on the spectroscopic properties of Lys96Arg UMC is almost identical to those of the WT protein, and thus this residue is also not responsible for the alkaline transition [308]. The most likely cause of the alkaline transition (see Fig. 21) is the $N^{\varepsilon 2}H$ moiety of one of the His ligands (in all cupredoxins the His ligands coordinate via their $N^{\delta 1}$ atoms) [308]. This is consistent with active site alterations which occur in RST at alkaline pH (p$K_a$ ∼ 9) and have been associated [309] with the deprotonation of the $N^{\varepsilon 2}H$ of the exposed His143 ligand.

The unusual features of phytocyanins (as cupredoxins) have been investigated and the results suggest that these molecules are involved in inter-protein ET [43]. However, the exact physiological function of the phytocyanins, possibly one of the largest families of plant proteins, remains unknown. Structural and spectroscopic studies of phytocyanins have highlighted the unusual active site properties of the STCs where an axial Gln ligand coordinates in a monodentate fashion via its $O^{\varepsilon 1}$ atom [43,46,161,175]. The influence of this natural active site modification on the

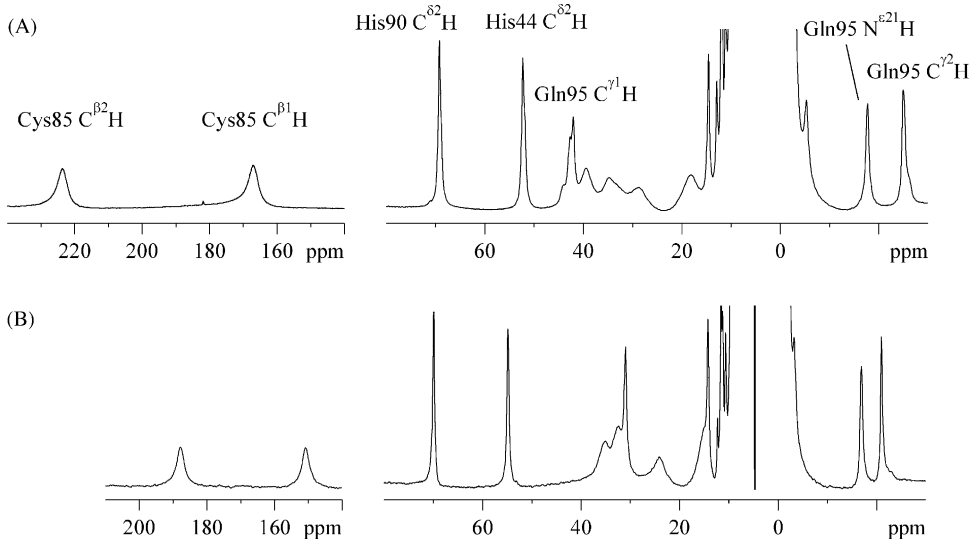



Fig. 20. $^{1}$H NMR spectra (300 MHz) of Ni(II) UMC at 30 °C in 90% $H_2O$/10% $D_2O$ [307]. The spectrum in (A) was obtained at pH 8.0 whilst that in (B) was at pH 10.7. Some of the assignments made are included [175].

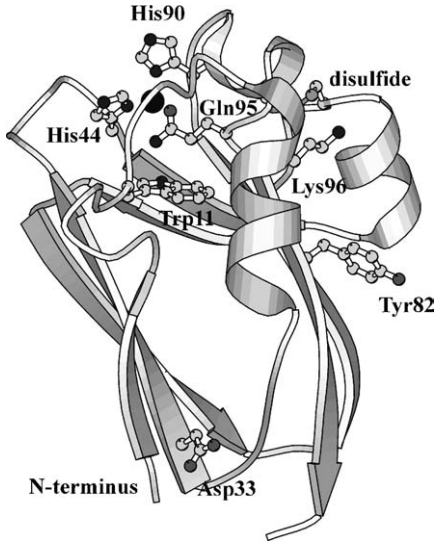



Fig. 21. The structure of UMC [43] in which the Cu(II) ion is shown in black and the side-chains of the coordinating amino acids are included. The residues which are potential causes of the alkaline transition are indicated as is the disulfide bridge close to the active site [308].

ET reactivity of cupredoxins will be discussed in the next section.

## 8. Active site engineering of cupredoxins

### *8.1. Site-directed mutagenesis*

The first cupredoxin genes were cloned almost 20 years ago [310–313] which lead to site-directed mutagenesis being utilised to investigate the relationship between their structure and function, with the initial focus being the unique properties of their T1 copper sites. These studies indicated that the Cys is the only ligand essential for a site with T1 spectroscopic properties [314,315]. Mutation of the other coordinating residues usually has a more subtle effect on the spectroscopic attributes of the site [316–319]. In particular, a plethora of axial ligand mutations have been published and in most cases the variant possesses T1 properties [123,170,297,316,320–322] (T1.5, i.e. between T1 and T2, in some cases [316,323]). In recent years T1 mutations have also been analysed in NiR [324–327] and the LACs [328–332]. The influence of T1 site mutations on ET reactivity has been assessed in very few cases.

The effect of making physiologically relevant active site mutations on the ET reactivity of the phytocyanins has been studied [269,270]. The Gln95Met UMC variant has a $k_{ese}$ of $1.0 \times 10^5\,M^{-1}\,s^{-1}$ whilst that of the WT protein is $1.8 \times 10^4\,M^{-1}\,s^{-1}$. Thus, the replacement of the strong Cu–O(Gln) bond with a weaker axial Cu–S(Met) interaction results in a site with significantly enhanced ET reactivity. This indicates that a site with an axial Gln has a $\lambda_i$ value larger than a T1 centre with an axial Met ligand which is consistent with the differences seen in the crystal structures of Cu(II) and Cu(I) proteins [43,60,63,77]. The Gln95Met UMC mutation results in a 130 mV increases in $E_m$. In other cupredoxins similar mutations result in 35–160 mV changes in the $E_m$ [123,170,320–322]. The influence of physiologically relevant mutations in the phytocyanins indicates that the axial position at a T1 site can tune $E_m$ [125] for reaction with a particular partner without significantly compromising ET.

Recent spectroscopic investigations of CST, and its Gln99Met and Gln99Leu variants, have provided information about active site differences between the $Cu^{II}$ and $Cu^{I}$ proteins [123]. The 0.5 Å increase in the Cu–O(Gln99) bond length in the WT protein upon reduction [299] seems to be accompanied by a minimal alteration in the Cu–S(Cys89)

interaction. In the Gln99Met variant there is a slightly larger change in the Cu–S(Cys89) bond and it is assumed that there is very little alteration in the Cu–S(Met99) interaction (as seen for native cupredoxins with an axial Met ligand) which results in similar $\lambda_i$ values for the two proteins [123]. The absence of an axial ligand in the Gln99Leu CST mutant results in a shorter Cu(II)–S(Cys89) bond which lengthens further upon reduction than in WT CST [123]. The ET reactivity of such three-coordinate variants would provide information on the physiological reactivity of analogous sites naturally found in certain phytocyanins [50,69,70], LACs [68,71], Fet3p [14] and ceruloplasmin [13].

Although a large number of site-directed mutagenesis studies have focussed on the active sites of cupredoxins, the role of residues in the second coordination sphere has not been investigated in any detail. A few studies have looked at the hydrogen bonding interactions around the active site, concentrating on the residue adjacent to the N-terminal His and also the amino acid two after the Cys ligand (the NH of both form hydrogen bonds to the thiolate sulfur of this ligand) [220,221,333–337]. In all cases the hydrogen bonds prove to be essential for the stability of the active site. The mutation of the Pro residue two after the Cys ligand in AMI [221] and PAZ [335] results in the formation of the second hydrogen bond to the thiolate sulfur of the Cys ligand in these cupredoxins and causes a large increase in the $E_m$ [220,221,335] (and in AMI this mutation alters the p$K_a$ of the C-terminal His ligand—see Section 5). The role of aromatic residues in the second coordination sphere has been investigated [213,223]. The Met16Phe PAZ mutation, which introduces a π–π interaction between the Phe and the imidazole ring of the His81 ligand analogous to that seen in *D. crassirhizoma* PC (see Fig. 8), is of particular note [213]. As well as influencing the p$K_a$ for the His81 ligand in Cu(I) PAZ (see Section 5) this mutation results in a 60 mV increase in the $E_m$ probably due to increased hydrophobicity at the active site. Furthermore, the Met16Phe variant has a $k_{ese}$ which is almost three times larger than that of WT PAZ. This has been assigned to a lower λ value due to additional rigidity at the active site, although enhanced homodimer formation cannot be discounted [213]. More second coordination sphere mutants need to be studied to truly appreciate the active site architecture and functionality of cupredoxins.

### 8.2. Loop-directed mutagenesis

Many metal sites in proteins are fabricated from loop regions and this is the case for T1 copper centres whose architecture involves three ligands on a loop linking β-strands 7 and 8 (see Figs. 7 and 22), and the fourth coordinating residue on the loop linking β-strands 3 and 4. In some cases biological metal sites can be modeled using loops alone [338,339], but this is not possible in many cases including the cupredoxins [340]. The loop usually needs to be attached to a stable structure and loop-directed mutagenesis can be used to graft active sites onto protein scaffolds [341]. The cupredoxins possess

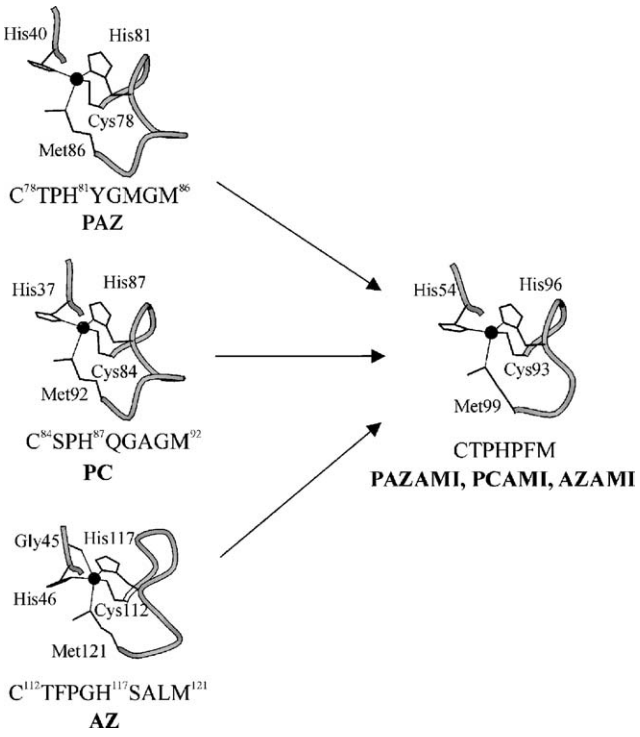



Fig. 22. The active site structures of *A. cycloclastes* PAZ [60], spinach PC [185], *P. aeruginosa* AZ [62,63] and *P. versutus* AMI [65]. The sequences of the C-terminal active site loops are shown and indicate the loop-contraction mutations that have been made to PAZ, PC and AZ to produce PAZAMI, PC AMI and AZAMI, respectively. Reprineted from [219] © 2005 American Chemical Society.

a rigid β-barrel structure which is an ideal scaffold for protein engineering studies, and loop-elongation mutagenesis has been used to modify the T1 copper site [207,216,217], and to introduce a dinuclear $Cu_A$ centre [342,343]. Loop-contraction mutagenesis can also be carried out and the loops of PAZ, PC and AZ have been replaced with the short sequence from AMI (see Fig. 22) [218,219]. In all cases these mutations have a limited effect on the spectroscopic properties (see Figs. 23 and 24) and hence active site structures of the loop variants. Phe114, whose backbone NH forms the second hydrogen bond with the thiolate sulfur of the Cys112 ligand in AZ, is replaced by Pro114 in AZAMI and thus this interaction is missing (as in AMI, PAZ and PC). The alterations [219] in the UV–vis (see Fig. 23) and EPR (see Fig. 24) spectra of Cu(II) AZAMI compared to AZ can be assigned to the missing hydrogen bond [9]. The presence of only a single hydrogen bond to the Cys enhances the electron density on the thiolate sulfur and results in increased spin density on the axial Met ligand.

Loop contraction always results in a decrease in $E_m$ (at ∼neutral pH) and the magnitude of this effect ranges from ∼30 mV in AZAMI to ∼60 mV in PCAMI [219]. AMI has the lowest $E_m$ of all of the WT cupredoxins used and thus the introduction of its C-terminal active site loop into PAZ, PC and AZ results in a $E_m$ which is closer to that of AMI.

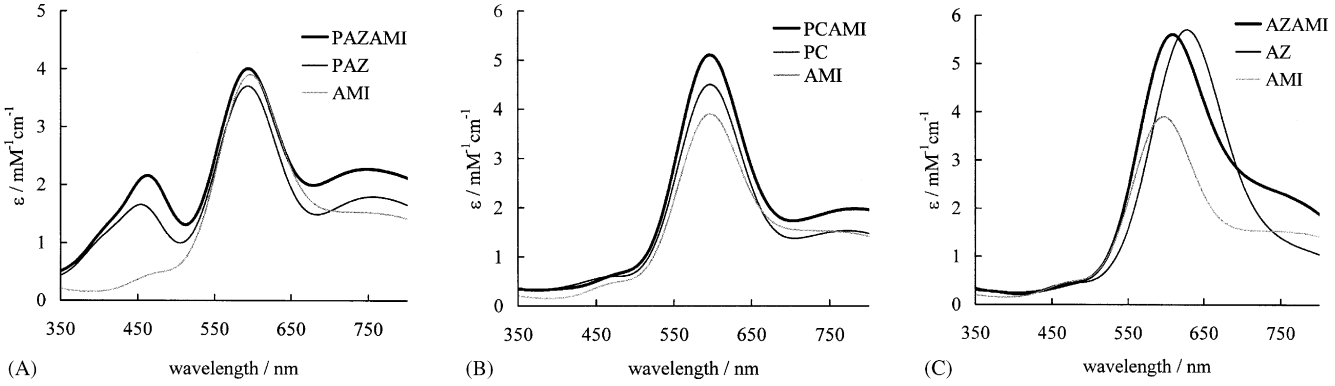



Fig. 23. UV/vis spectra of Cu(II) proteins at 25 °C in 10 mM phosphate pH 8.0: (A) PAZ, PAZAMI and AMI, (B) PC, PC AMI and AMI and (C) AZ, AZAMI and AMI [219].

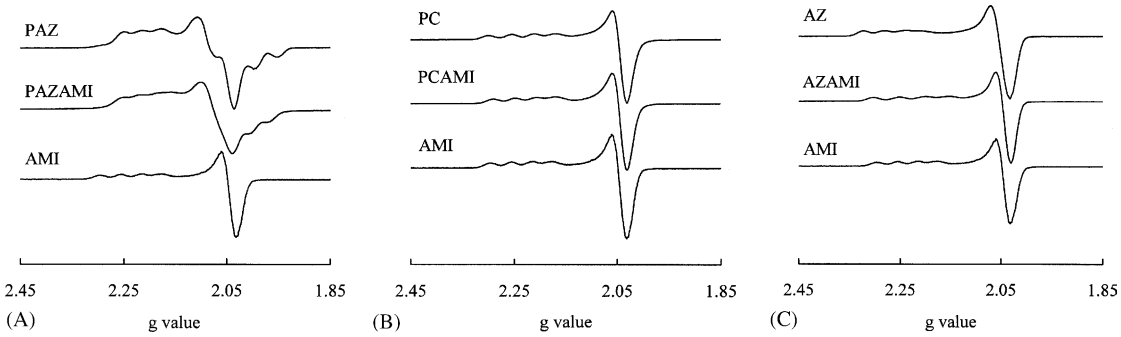



Fig. 24. EPR spectra of Cu(II) proteins at −196 °C in 25 mM Hepes pH 7.6 (40% glycerol): (A) PAZ, PAZAMI and AMI, (B) PC, PC AMI and AMI and (C) AZ, AZAMI and AMI [219].

Similarly, the loop elongation experiments on AMI, in which the active site loops of PAZ, PC and AZ are introduced, all result in an increase in $E_m$ to a value closer to that of the protein whose loop is used [216]. Thus, loop-contraction provides an active site environment preferable for the cupric ion, whereas loop elongation leads to an active site which favours Cu(I). The ET reactivity of the loop-contraction variants has been assessed by determining their $k_{ese}$ values [219]. The largest effect is seen in PAZ where an eight-fold decrease in $k_{ese}$ is observed upon loop contraction. PCAMI has a $k_{ese}$ which is only half that of PC whereas in AZ loop contraction has almost no influence on ET reactivity. Thus, most cupredoxins have scaffolds that can accommodate the shorter loop of AMI without significantly influencing ET reactivity. Loop elongation in AMI always resulted in a decreased $k_{ese}$ although the proteins are still capable of supporting ET [216,217].

The loop contraction studies show that a shorter ligand-containing loop at a T1 copper centre results in an environment which stabilises Cu(II) over Cu(I) [218,219]. The loop elongation studies demonstrate that in AMI a longer loop leads to a site which has a preference for Cu(I) [207,216,217]. For many years it has been suggested that the active site structure of a cupredoxin is more suited to Cu(I) rather than oxidised copper, thus explaining the elevated $E_m$ values for these proteins compared to the Cu(II)/Cu(I) aqua couple [76,344]. Studies on denatured AZ have shown that the folded cupredoxin domain of this protein actually stabilises Cu(II) [92,345,346]. The preference for oxidised or reduced copper is not only controlled by the β-barrel scaffold and the ligating residues of a cupredoxin but is also dependent on the C-terminal ligand-containing loop. The loop-contraction and loop-elongation variants possess active sites which demonstrate preferences for Cu(II) and Cu(I), respectively, yet they are still entatic in that they support fast intermolecular ET. Further studies are required to truly understand the importance of the loop and the scaffold onto which it is grafted for the structure and functionality of T1 copper sites.

## 9. Concluding remarks

The work described in this review highlights the complexity of ET proteins. As further information becomes available a more detailed understanding is developed which can be applied to the appreciation of complex enzymes possessing cupredoxin domains, and also to the development of potential applications of these molecules. However, there are many questions that still need to be answered to truly appreciate the ET reactivity of cupredoxins.

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