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Impact of copper ligand mutations on a cupredoxin with a green copper center

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Mononuclear cupredoxins contain a type 1 copper center with a trigonal or tetragonal geometry usually maintained by four ligands, a cystein, two histidines and a methionine. The recent discovery of new members of this family with unusual properties demonstrates, however, the versatility of this class of proteins. Changes in their ligand set lead to drastic variation in their metal site geometry and in the resulting spectroscopic and redox features. In our work, we report the identification of the copper ligands in the recently discovered cupredoxin AcoP. We show that even though AcoP possesses a classical copper ligand set, it has a highly perturbed copper center. In depth studies of mutant's properties suggest a high degree of constraint existing in the copper center of the wild type protein and even the addition of exogenous ligands does not lead to the reconstitution of the initial copper center. Not only the chemical nature of the axial ligand but also constraints brought by its covalent binding to the protein backbone might be critical to maintain a green copper site with high redox potential. This work illustrates the importance of experimentally dissecting the molecular diversity of cupredoxins to determine the molecular determinants responsible for their copper center geometry and redox potential.

1. Introduction

Type 1 (T1) copper proteins are widespread, copper-containing polypeptides involved in monoelectron transfer processes. They play critical roles in various biological processes, such as respiration, photosynthesis and nitrogen metabolism. Despite an overall low level of sequence identity, which explains the difficulty to identify them and predict their properties, T1 copper proteins contain one copper atom and share a common fold, the so-called cupredoxin fold, that consists of a rigid Greek-key β-barrel [1–2]. T1 copper sites can be found in single-domain cupredoxins, such as azurin, rusticyanin and plastocyanin, or as part of multidomain cupredoxins [3–4]. The latter include copper-containing nitrite reductases (Nirs) and multi-copper oxidases (MCOs), such as laccase. The known crystal structures of T1 copper proteins show that the copper atom is usually tetracoordinated. Three strong equatorial ligands lie on a trigonal plane, and correspond to the imidazole and thiolate moieties provided by two histidine and one cysteine residues, respectively. The fourth, weakly bound axial ligand is usually provided by the thioether sulfur of a methionine residue and in some cases by glutamine residue, and leads to tetrahedral or tetragonal geometries [5–7]. Extensive site-directed mutagenesis studies, together with the identification of diverse members of the cupredoxin superfamily, have clearly demonstrated that natural or designed modifications, that affect these ligands or the close environment of the copper center, can lead to drastic changes of the metal site geometry (from tetrahedral to tetragonal) and of the resulting spectroscopic and redox features [8–15]. Based on these differences, four groups have been distinguished: the blue, the perturbed-blue, the green and the red copper sites [6,16]. Also called “classical T1”, blue copper sites are characterized in their cupric form by an intense absorption peak ($\varepsilon \geq 4000\ M^{-1} \cdot cm^{-1}$) at around 600 nm in their UV–Visible absorption (UV–Vis ABS) spectra, which is responsible for their characteristic blue color. This absorption band is mainly due to the strong Cu^{2+}-S(Cys) covalent bond, that generates an intense S(Cys) $\pi \rightarrow$ Cu ligand-to-metal charge transfer (LMCT) transition [16–17]. In electron paramagnetic resonance (EPR), a characteristic axial spectrum with a small hyperfine

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splitting constant ($A_{//} > 80 \times 10^{-4}$ cm^{-1}) is observed [18]. Perturbed-blue copper sites, as found in rusticyanin, pseudoazurin and cucumber basic protein (CBP), result into rhombic EPR spectra and into an additional UV–Vis ABS band near 450 nm. The latter has been mainly attributed to S(Cys) $\sigma \rightarrow Cu$, and in some case to S(Met) $\rightarrow$ Cu LMCT transitions. Spectroscopic and DFT studies demonstrate that perturbed-blue copper sites are due to a stronger interaction between the copper and the axial ligand, that leads to a small release of the copper-to-cysteine bond and consequently to a distorted tetrahedral geometry [16]. An even stronger Cu^{2+}-S(Met) bond is found in some green copper sites. Also called T1.5, these metal centers have been rarely found in single-domain cupredoxins, and one of the best studied systems corresponds to the cupredoxin domain of Nir from Rhodobacter sphaeroides. The Nir copper site has a highly distorted tetrahedral geometry, accompanied by an increase in the intensity of the band near 450 nm over the 600 nm band (the A_{450}/A_{600} ratio also called R_L value) and responsible for the green color of the oxidized protein [16, 19–20]. Finally, only one example of red copper site, called "T2-like", has been found to date in cupredoxins. This is the metal center of nitrosocyanin, which is characterized by a strong UV–Vis ABS band at 385 nm [21–22]. This band has been attributed to a S(Cys) $\sigma \rightarrow$ Cu LMCT transition. In contrast with blue and green copper sites, the nitrosocyanin EPR spectrum is characterized by large hyperfine splitting ($A_{//} \sim 150 \times 10^{-4}$ cm^{-1}). Such a behavior has been attributed to the unique structure of the T2-like center due to a different ligand set: with a strong metal-ligand axial interaction (histidine instead of methionine), a weak metal-ligand equatorial bond (glutamate instead of histidine) and an additional equatorial coordination to a water molecule. This leads to a tetragonal copper site with unique spectroscopic features [16,22–23].

Like in the case of nitrosocyanin and its unusual coordination site, the study of natural and mutated copper-binding proteins has shown that the nature of the copper ligands plays a critical role in controlling the electronic structure, the spectroscopic properties and the redox potential of copper sites. Site-directed mutagenesis of cupredoxins demonstrates that the substitution of one of the copper ligands can drastically modify their spectroscopic behaviors and/or their redox properties. The cysteine residue, for example, is known to be absolutely required for copper binding [24–25]. Mutations of axial methionines to a stronger ligand, such as histidine or glutamine, has been shown to turn a blue copper sites into green ones [10,13,26–32]. On the contrary, in the case of Nir, the replacement of the axial methionine with a weaker threonine ligand, converts the green copper site into blue [27,33]. Several studies have also revealed that modifications of the axial ligand, not only perturb the geometry of the metal center, but drastically change the redox potential too [27]. In a study from Berry and co-workers, the replacement of Met121, the axial ligand of azurin, with unnatural amino acids revealed that hydrophobicity is of outmost importance in tuning the reduction potential of T1 copper proteins [34–35]. Finally, unusual copper center properties may arise from ligand substitution, as observed in two recent examples of natural cupredoxins: Auracyanin D, a single-domain green cupredoxin with a glutamine instead of the usual axial methionine, resulting into a highly distorted tetrahedral geometry and a green copper center different from the one from NiR [36–37]. The second example is a purple cupredoxin, Nmar1307, which has been recently described [38]. Found in the archeon Nitrosopumilus maritimus, it contains a monuclear copper site with a classical set of equatorial ligands, a water molecule as the axial ligand and potential enzymatic activity, extending the range of activities carried out by this class of proteins [38].

Despite the wealth of information resulting from comparative and mutagenesis studies, the finding of copper binding sites with novel proteins brings new insights and novel questions that challenge our knowledge of copper-binding proteins and prompts for in depth study of cupredoxins diversity. The protein AcoP from Acidithiobacillus ferrooxidans [39] is a single-domain cupredoxin that we recently discovered in tight interaction with the cytochrome c oxidase and characterized [40–41]. We have demonstrated that AcoP has a green copper site and exhibits a strikingly high redox potential (566 mV at pH 5 vs SHE) [40], compared to the usually low values reported for the green copper sites characterized to date (90–250 mV at pH 7 vs SHE) [19, 36]. In this study, we determine AcoP copper-ligands and measure the impact of their substitution on the spectroscopic and redox properties of AcoP metal center.

The comparison of copper-binding signatures, and the recent finding of unusual axial ligands in non-canonical cupredoxins (Fig. 1), reinforced our doubts concerning the assignment of AcoP axial residue, choosing between an adjacent methionine and histidine, and that of the N-terminal copper-binding histidine. We show that wild-type and mutant AcoP have a classical ligand set, yet anomalous spectroscopic and redox properties. In depth study on this new model cupredoxin with interesting properties should further help in the identification of the critical determinants that tune a metal center geometry and redox potential.

2. Results

2.1. Copper-coordinating ligands of AcoP and characterization of the respective mutants

Sequence alignment based on predicted secondary structure elements with cupredoxins of known 3D structure [40] suggested that AcoP possesses a typical copper-binding ligand set involving one cysteine (Cys159), two histidines (His85 and His166) and one axial methionine (Met171). However, the low percentage of sequence identity with cupredoxins of known structure, doesn't allow unambiguously attribution of His85 as the N-terminal ligand of the copper-ligand signature. More important, the unusual spectroscopic and redox properties of AcoP could be accounted for by an axial ligand different from a methionine, as recently reported for the non-canonical copper-ligand sets observed in Auracyanin D and Nmar1307. The presence of a His residue in position 172, i.e., next to Met171, suggests that this could be the case.

Given the unusual combination of redox and spectrocoscopic properties of AcoP and its potential to become a model system for a better understanding of the copper site properties in cupredoxins, we decided to confirm the identity of the copper ligands by mutating all of them to alanine. The double M171A-H172A mutant was also generated, resulting in a total of six AcoP mutants (Table 1).

All proteins were produced in E. coli, purified to homogeneity in their apo form and supplemented with exogenous Cu^{2+} after purification, following the same procedure as described for the wild-type protein (AcoPwt). The effect of each mutation on the AcoP copper content

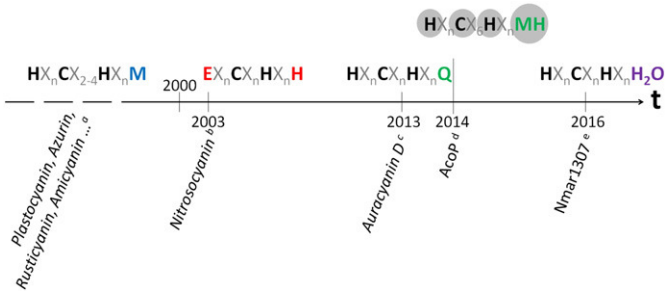

Fig. 1. Amino acid identity of copper binding sites in some single domain cupredoxins. The color code corresponds to the equivalent color for the copper center geometry (blue, green, red or purple). Before year 2000, several cupredoxins have been discovered harboring for the majority a blue color and the copper ligand depicted on the scheme (few exceptions exist, like Stellacyanin and Umecyanin which possess a glutamine in axial position and have a strongly perturbed copper center [7,12]. Since 2000, novel cupredoxins with different copper ligands has been discovered: [a] [60], [b] [23], [c] [36–37], [d] [40], [e] [38]. Grey circles highlight the hypothetical copper-binding signature in AcoP, corresponding to His85, Cys159, His166 and either Met171 or His172.

Table 1
List of AcoP mutants designed and characterized in this study.

Protein name	Sequence
AcoP wt	H_{85} X_n C_{159} H_{166} M_{171} H_{172}
AcoP H85A	A_{85} X_n C_{159} H_{166} M_{171} H_{172}
AcoP C159A	H_{85} X_n A_{159} H_{166} M_{171} H_{172}
AcoP H166A	H_{85} X_n C_{159} A_{166} M_{171} H_{172}
AcoP M171A	H_{85} X_n C_{159} H_{166} A_{171} H_{172}
AcoP H172A	H_{85} X_n C_{159} H_{166} M_{171} A_{172}

was determined using Inductively Couple Plasma-Optical Emission Spectroscopy (ICP-OES) (Table 2). Each mutant contained equimolar Cu except for the C159A construct. This result confirms that only the cysteine residue is essential for copper binding, in agreement with mutagenesis studies on other cupredoxins [24,43]. The impact of each mutation on the overall fold of AcoP was assessed by far-UV Circular Dichroism (CD) spectroscopy. As shown in Fig. 2, similar spectra were recorded for wild-type and mutant AcoP constructs, with a common minimum around 217 nm that suggests a fold rich in β-strands, consistent with the predicted cupredoxin fold for these proteins. In the case of the C159A mutant and of apo-AcoP, however, a small spectral variation was observed (Fig. 2, inset), suggesting that for both constructs, copper depletion partially affects the secondary structure of AcoP. In line with this, recent studies have highlighted that a high degree of conformational heterogeneity exists in the metal-binding region of some apo-cupredoxins [44]. Vila and coll. proposed that copper binding is a major determinant of copper-site structure rigidity and a major determinant of high electron transfer efficiency in cupredoxins [44–45].

Although mutation of the other AcoP putative ligands does not impair copper binding, it has a profound impact on their spectroscopic features. Replacing H85 or H166 by alanine does not allow recording any spectra in the visible range (Fig. 3). Even after the addition of an excess of the strong oxidizing agent K_2IrCl_6, the samples are still colorless. Accordingly, whereas a well-defined redox wave centered at 0.56 V vs SHE was recorded for AcoPwt at a pyrolytic graphite (PG) electrode, witnessing of a redox-competent center, no electrochemical signals were detected in a potential window from − 100 to + 800 mV for the mutant (Fig. 4). Since the two mutants bind copper, as assessed by ICP-OES (Table 2), the absence of redox and spectroscopic features suggests that their copper site is maintained in a reduced form, at least in the conditions tested. A similar behavior has been observed for the H145A mutant of Nir from Alcaligenes faecalis, and the H46G and H117G mutants of azurin from Pseudomonas aeruginosa [46–47]. In these systems, histidine mutation by a small non-coordinating residue (Gly or Ala) leads to a three-coordinated copper site, to the stabilization of the cuprous state due to S-donor ligation and to a strong increase of the redox potential. This contrast with the replacement of His residues by an Asp that partially retains the metal site features [48]. For Nir and azurin, histidine mutation gives rise to an additional cavity in the vicinity of the metal site. In this case, the addition of exogenous ligands, such as imidazole which chemically mimics histidine, provides a fourth coordination bond to the active site copper, and results in lower and measurable redox potential compared to the mutant [47]. Further on, for these mutants, depending on the chemical nature of the exogenous ligand added, spectroscopic features typical of blue, green or red copper sites can be observed [46,49–52]. In contrast to these results, the reconstitution of a four-coordinated copper site in the H85A and H166A mutants failed, and the addition of excess imidazole in all conditions tested, even in a highly oxidizing environment, did not allow us to retrieve any spectroscopic and electrochemical features reminiscent of a T1 copper site.

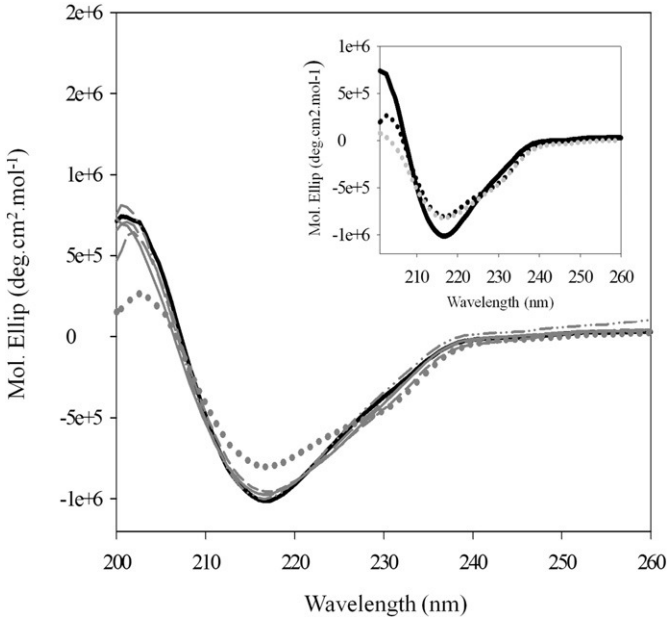

Fig. 2. Modification of AcoP secondary structure after point mutations. Far-UV CD spectra of purified holo-AcoPwt (——) and the H85A (— —), C159A (·····), H166A (——), M171A (– – –) and H172A (— · ·) mutants. Inset: Superposition of the far-UV CD spectra of holo- and apo-AcoPwt (·····), as well as of the C159A mutant, shows differences in the secondary structures content between the holo (AcoPwt) and the apo forms (apoAcoP or C159A mutant) of AcoP suggesting that copper binding may play a role in microdomain folding. All spectra are recorded in buffer 50 mM sodium acetate at pH 5.0.

Finally, mutations of Met171 and His172 were introduced in order to unveil the identity of the axial copper ligand in AcoP. The spectrum of the H172A mutant nicely superposes to the one recorded for AcoPwt, with an intense and a weak band at 438 nm and 568 nm, respectively, and a broad peak around 690 nm (Fig. 3), suggesting that His172 is not one of the copper ligands. For the M171A mutant, instead, a strong spectral change is observed, with a strong band at 368 nm and two minor bands at 455 and 630 nm (Fig. 3). These novel spectral features are reminiscent of a red copper site, and imply that Met171 is the fourth axial copper ligand, being essential in defining the electronic structure of this metal site. Based on what is known for nitrosocyanin red copper site, where a His occupies the axial position, we reasoned that the nearby His172 could replace the axial methionine in the M171A mutant and lead to the observed red copper site (Fig. 1). To test this hypothesis, we constructed the double mutant AcoP M171A-H172A. The copper content and the spectroscopic features of the AcoP M171A and the AcoP M171A-H172A mutants were very similar. We can thus conclude that His172 is neither the axial copper ligand in AcoPwt, nor it is involved in the formation of the red copper site in the AcoP M171A mutant.

These results allow us to propose that the metal binding site in AcoP is defined by canonical T1 ligands: His85, Cys159, His166 and Met171. Therefore, the perturbed nature of its electronic structure cannot be explained by an atypical ligand set as found for example in Auracyanin D or Nmar1307 (Fig. 1).

Table 2
Copper content by ICP-OES of holo and apo-AcoPwt, and AcoP mutants.

	AcoPwt apo	AcoPwt holo	AcoP H85A	AcoP C159A	AcoP H166A	AcoP M171A	AcoP H172A
Cu/protein	0.16 ± 0.11	0.96 ± 0.16	0.92 ± 0.24	0.11 ± 0.06	1.12 ± 0.25	1.00 ± 0.26	1.14 ± 0.15

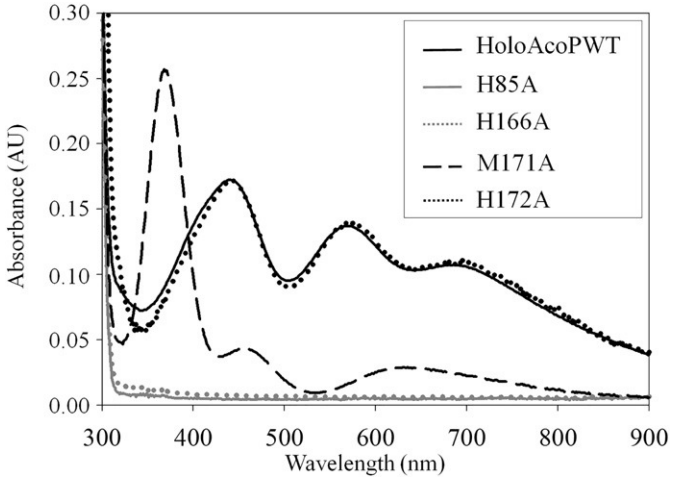

Fig. 3. Spectroscopic properties of AcoP mutants. Room temperature UV–Vis ABS spectra of 50 μM purified holo-AcoPwt (——) and AcoP H85A(——), H166A (····), M171A (– – –) and H172A (·····) are recorded in 50 mM sodium acetate buffer containing 0.005%DDM and 5%glycerol, at pH 5.0 after addition of K_2IrCl_6.

2.2. Spectroscopic characterization of AcoP M171A mutant

To gain further insight into the electronic structure of the copper site in the M171A mutant, UV–Vis ABS and Vis-CD spectra were collected and compared to the ones obtained for AcoPwt. Spectra from both variants were deconvoluted. Gaussian resolution obtained from these fits is shown in Fig. 5 and Energies and ε values for the absorption bands are summarized in Table 3. Band assignment was made on a comparative bases with previous studies on blue, green and red copper site (plastocyanin, Nir and nitrosocyanin, respectively) [20,22]. The absorption spectrum of AcoPwt is characterized by several typical bands. One, found in the low energy region at 700 nm (band 1), likely corresponds to the copper d-d transition band. Three more bands found in the high energy region, at 563 nm (band 2), 451 nm (band 3) and 394 nm (band 4), are compatible with S(Cys) → Cu π, S(Cys) → Cu σ and S(Met) → Cu LMCT transitions, respectively (Fig. 5 A, Table 3) [20]. AcoP M171A exhibits different spectroscopic features than the wild type (Fig. 5B, Table 3). In analogy with what is observed for the red copper site in nitrosocyanin, its spectrum is characterized by three bands at 625 nm (band 1), 456 nm (band 2) and 368 nm (band 3), assigned to a d-d, S(Cys) → Cu π and S(Cys) → Cu σ LMCT transitions, respectively. In red copper sites, the cysteine-to-copper LMCT transitions (S(Cys) → Cu π and S(Cys) → Cu σ) are shifted to higher energies in comparison to those in blue and green copper sites. In addition, the higher energy transition (S(Cys) → Cu σ) is ~3 to ~10 fold more intense than its counterpart in green and blue copper sites, respectively [22]. This is associated with a strong increase of the R_L value from ~3 in nitrosocyanin, to ~6 in the case of AcoP M171A.

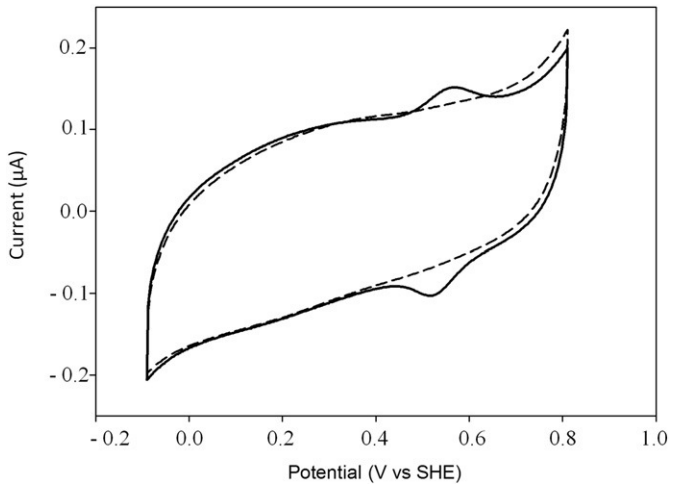

Fig. 4. Typical cyclic voltamograms of AcoPwt and AcoP mutants. 50 μM AcoPwt (——) and the H166A mutant (– – –) were deposited at the PG membrane electrode in 10 mM sodium acetate buffer pH 5.0 ($v = 10\ mV.s^{-1}$). Fully superposable voltamograms were also recorded for AcoP H172A (——) and AcoP H85A (– – –).

The red copper nature of the active site in the M171A mutant is further supported by EPR spectroscopy. The X-band EPR spectrum of AcoP M171A is shown in Fig. 6 A. While AcoPwt displays EPR features typical of a green copper site [40], the M171A mutant spectrum and hyperfine splitting are very similar to the ones observed for nitrosocyanin red copper site (Table 4). Plotting the $g_{//}$ and $A_{//}$ values of the M171A mutant into a Peisach-Blumberg diagram which correlates these two parameters for mononuclear copper sites, also reveals that the EPR signature of this mutant fits well with those of nitrosocyanin red copper site (Fig. 6B) [53]. To conclude, the spectroscopic data from EPR, UV–Vis ABS and Vis-CD analysis of AcoP M171A are indicative of a tetragonal geometry in analogy to what is observed for the red copper site of nitrosocyanin [22]. After purification, AcoP M171A was fully oxidized protein. Even upon reduction with a strong reductant such as dithionite, no change in the UV–Vis ABS spectrum could be observed. This result suggests that although nitrosocyanin and the M171A mutant share similar spectral features, the copper atom in AcoP M171A is constrained into the oxidized state. Mutation of the axial methionine in other cupredoxins is known to lead to strong modifications of the redox potential [13,25,31,54], however AcoP M171A has an extreme behavior with a very low redox potential that cannot be experimentally determined.

2.3. Temperature and pH behavior of AcoP M171A

In order to assess the impact of Met171 substitution on the intrinsic properties of the copper site, we next decided to investigate the behavior of this mutant upon temperature and pH variations. Previous studies on AcoPwt have revealed a stable copper site, showing no changes in spectroscopic properties upon temperature or pH variations [40]. As shown in Fig. 7 A, the low temperature UV–Vis ABS spectrum of AcoP M171A is characterized by a strong absorption band at 364 nm, a weak absorption band at 450 nm and a broad band at 630 nm with a R_L value of ~3.2 (vs R_L~6 for the room temperature UV–Vis ABS spectrum). The two-fold decrease in the R_L value reveals modifications in the copper environment upon temperature variations. A similar trend in temperature dependence of UV–Vis ABS spectra has been previously reported for the green Nir from R. sphaeroides and attributed to an equilibrium between two electronic states of the metal site at room temperature, reflecting an unconstrained T1 copper site [55]. The absorption features of AcoP M171A in the UV–Vis were also sensitive to pH changes (Fig. 7 B), since they disappear at pH 2.5. Addition of extra copper did not restore the spectral features. These could be partially recovered by rising the pH to 5.0 with extra copper (Fig. 7 B, inset). In contrast with the metal site in AcoPwt, stable over a broad pH range, the pH sensitivity of AcoP M171A might be due to a deprotonation event in the vicinity of the copper site, resulting in copper loss.

Reconstitution at pH 5.0 of the holo form of AcoP M171A, after copper stripping at pH 2.5, leads to an important observation: the spectrum of AcoP M171A at pH 5.0 is significantly different in phosphate and acetate buffer (Fig. 8). To explain this behavior, we propose that copper axial ligation in this mutant is provided by exogenous molecules present in the buffer, and that their chemical nature affects the resulting spectra by change in the ligand set and, as such, the copper center geometry. Acetate may represent the fourth copper ligand in AcoP M171A prepared in acetate buffer. This is confirmed by the fact that the addition of acetate to the M171A mutant in phosphate buffer allows

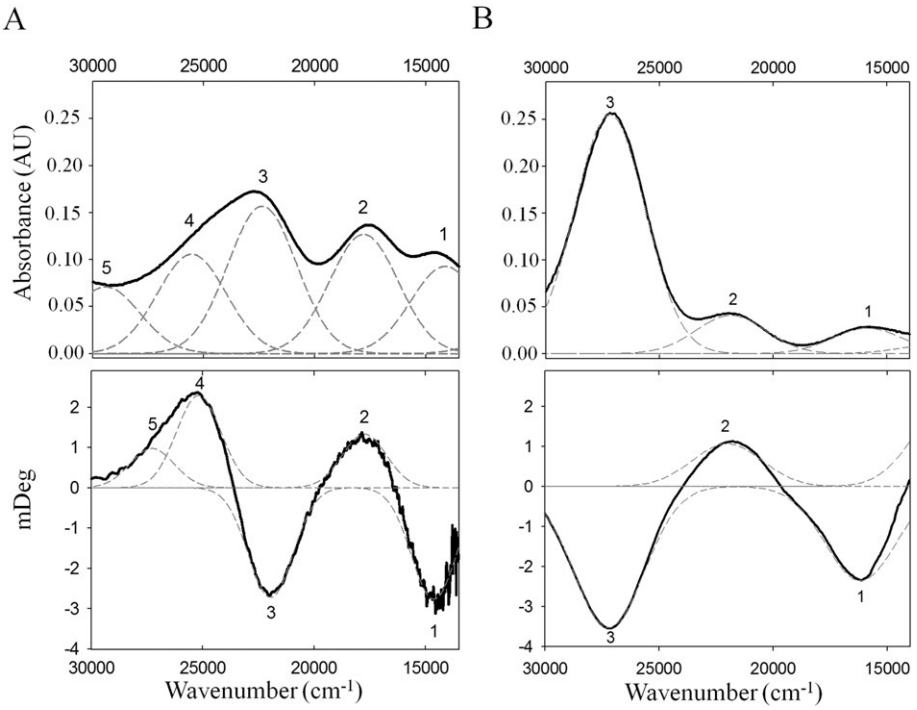

Fig. 5. Comparison of spectroscopic characteristics of AcoP M171A and AcoPwt. UV–Vis ABS spectra (top) and Vis CD spectra (bottom) of holo-AcoPwt (A) and AcoP M171A (B). Experimental spectra (black solid line) were recorded using 50 and 100 μM of proteins for UV–Vis ABS spectra and Vis CD spectra, respectively, in 50 mM sodium acetate buffer, containing 0.005% DDM and 5% glycerol, at pH 5.0. Gaussian resolution of bands (dashed grey lines) has been attributed using Peakfit. The numbering scheme is chosen to be consistent with the assignment of bands in plastocyanin (blue copper site), Ac Nir (green copper site) and nitrosocyanin (red copper site). Bands assignment is reported in Table 3.

the recovery of the initial spectrum of AcoP M171A purified in sodium acetate at pH 5.0, meaning that the acetate molecule binds with strong affinity the copper center of AcoP M171A. We have also seen that acetate must be removed by acidification at pH 2.5 for other ligands to bind to the mutant active site.

Overall, these experiments reveal that the axial M171 plays a critical role in constraining the copper site in wtAcoP. For AcoP M171A mutant, exogenous molecules present in the used buffers account for a fourth metal coordination and lead to spectral features typical of a T2 red copper center.

2.4. AcoP M171A copper site reconstituted by exogenous ligands

The pH sensitivity of the M171A mutant, described above, suggests that this mutation has created a solvent-accessible cavity in the first coordination shell of the metal site that might allow small molecules to bind copper. To test this hypothesis, we used several exogenous ligands, well known to bind copper in other cupredoxin mutants, and we tested the impact of their binding on metal site properties (Fig. 8) [27,56]. We investigated the effect of azide (N_3^-), thiocyanate (SCN^-), ethanol (to mimic Thr or Ser), imidazole (to mimic His) and dimethylsulfide (DMS, to mimic Met). While these molecules had no effect, as expected, onto the spectral features of oxidized AcoPwt (data not shown), they all affected the spectrum of AcoP M171A, apart from ethanol (Fig. 8). All the resulting absorption spectra are typical of a red copper site, although they feature different peak maxima and a wide range of R_L values that vary from 2.0 to 6.2 (Table 5). These differences illustrate different copper center geometries induced by exogenous ligand binding, which can be due to slight changes in the copper-to-ligand distances. Importantly, our data show that binding of DMS, which mimics the thioether of a methionine side chain, changes slightly the spectra but is not able to restore the green spectroscopic features of AcoPwt. Similarly, addition of ethanol did not mimic a weak ligand leading to blue spectroscopic features as red features are still measured. This result reveals that the chemical nature of the axial residue is not the unique determinant for green copper center geometry in AcoP, and that the rack effect elicited by the protein architecture may play a fundamental role.

In contrast to previous studies, where the redox potential could be fine-tuned by exogenous ligands, all tested molecules lead to species which cannot be reduced in the condition tested. The ligand-reconstituted M171A mutant is either fully oxidized and impossible to reduce (M171A with acetate), or it can be reduced irreversibly (other ligands, data not shown), meaning that the oxidized form could not be restored even after adding a strong oxidizing agent. To our knowledge, a similar behaviour has only been reported for the H46G and H117G variants of azurin. In these azurin equatorial mutants, the copper centre

Table 3
Experimental UV–Vis ABS and Vis CD spectroscopic parameters of AcoPwt and the M171A mutant compared to T1 copper centers of plastocyanin (Pc), nitrite reductase from Achromobacter cycloclastes (Ac Nir) and nitrosocyanin (Nc) [20,22].

		Energy (cm^{-1})					ε ($M^{-1}.cm^{-1}$)				
Band	Assignment	AcoP wt	AcoP M171A	Pc	Ac Nir	Nc	AcoP wt	AcoP M171A	Pc	Ac Nir	Nc
1	d-d	14,370	16,000	5000–13,950	5600–14,900	10,600–17,600	1800	520	250–1400	310–1160	430–810
2	S(Cys)π	17,762	21,930	16,700	17,550	20,350	2500	800	5160	1490	2200
3	S(Cys)σ	22,153	27,191	18,700	21,900	25,550	3100	5060	600	2590	7000
4	S(Met)	25,353	–	23,440	25,650	–	2000	–	250	1750	–
5	n.d.	28,339	–	–	–	–	1380	–	–	–	–

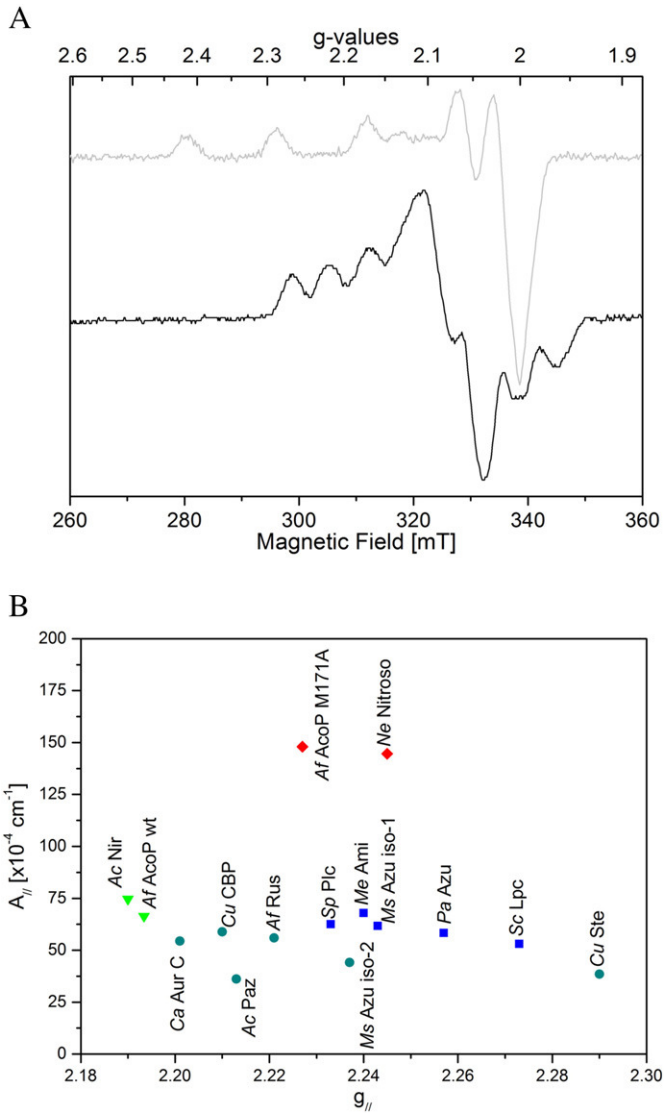

Fig. 6. EPR spectra of AcoP M171A and AcoPwt. (A) Frozen solution X band EPR spectra of 50 μM holo-AcoPwt (black) and AcoP M171A (grey) at pH 5.0 in 50 mM sodium acetate containing 0.005% DDM and 5% glycerol. Microwave frequency = 9.479 MHz. (B) Peisach-Blumberg diagram from [53].

was accessible to exogenous molecules, it could be reduced, but it was impossible to oxidize it back [52]. This behavior has not been fully understood yet, but it has been proposed that upon reduction, the fourth ligand, most likely a water molecule, dissociates from the copper atom. This would change copper coordination from tetra- to tri-coordinated, stabilize the Cu^{+} over the Cu^{2+} state and lead to an extreme increase in the redox potential.

Table 4
X band EPR parameters of AcoPwt and AcoP M171A.

	AcoPwt	AcoP M171A
g factor	gx = 2.019	gx = 2.020
	gy = 2.057	gy = 2.020
	gz = 2.193	gz = 2.227
$A_{//}$ ($\times 10^{-4}$ cm^{-1})	Ax = 65	Ax = n.d.
	Ay = 12	Ay = n.d.
	Az = 66	Az = 148

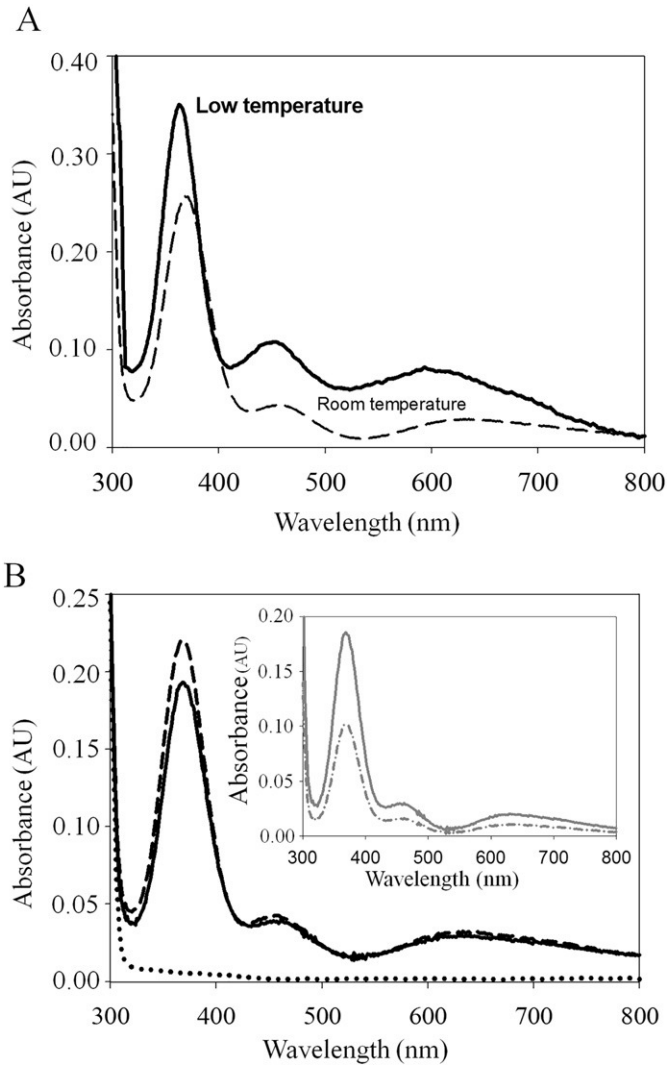

Fig. 7. Effect of temperature and pH on AcoP M171A spectroscopic properties. (A) UV–Vis ABS spectra of the same sample of AcoP M171A (50 μM) in 70%(v/v) glycerol and 50 mM sodium acetate buffer pH 5.0, were either recorded at room temperature (black dashed line) or at low temperature (~77 K, black solid line). (B) Room temperature UV–Vis spectra of AcoP M171A (50 μM) prepared in 50 mM acetate buffer pH 5.0 and subsequently dialysed against 20 mM phosphate buffer pH 5.0 (black solid line), pH 7.5 (black dashed line) and sodium phosphate-citrate buffer pH 2.5 (black dotted line). Inset: comparison of spectra of AcoP M171A in 50 mM acetate buffer pH 5.0, (grey solid line) and after double dialysis against phosphate buffer pH 2.5 and, subsequently, acetate buffer pH 5.0, in the presence of 2-fold molar excess of copper (grey dash-point line).

3. Discussion

We have previously shown that AcoP exhibits features that are unique for a single-domain cupredoxin, notably a green copper site coupled to an unexpectedly high redox potential [40]. In this work we define the copper ligand set by an extensive mutagenesis study of the putative metal ligands allowing us to discard that these unusual characteristics are due to a distinctive ligand set, as observed in Auracyanin D, for which the mutation of its axial Glutamine ligand to methionine leads to properties of classic blue cupredoxin [37]. Indeed, AcoP is characterized by a canonical T1 copper ligand set, defined in this case by residues His85, Cys159, His166 and Met171. To our knowledge, AcoP is the first single-domain green type cupredoxin with this ligand set. Similar green copper sites have only been found to date in some multi-domain cupredoxins from the Nirs family. Extensive studies on green nitrite reductases have shown that their spectroscopic features are due to a stronger axial Cu-S(Met) interaction than in classic T1 sites, and weaker

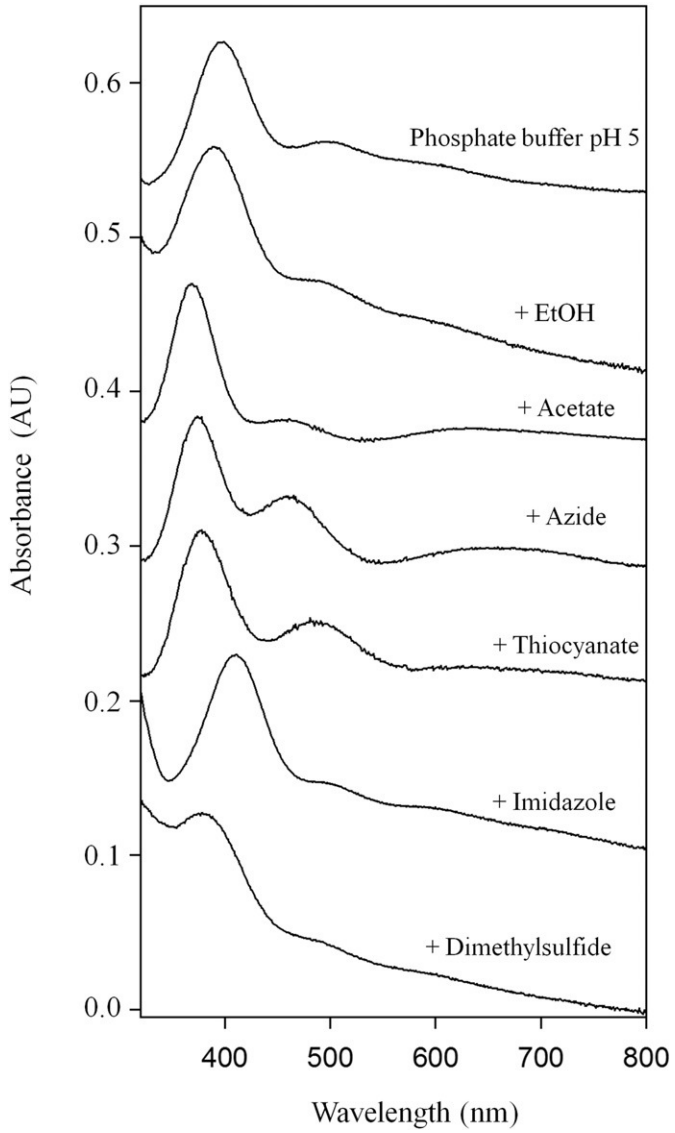

Fig. 8. Influence of exogenous ligand incorporation on the UV–Vis ABS spectroscopic features of AcoP M171A. AcoP M171A was first dialysed in sodium phosphate-citrate buffer pH 2.5 in order to remove the acetate initially bound to the protein. AcoP M171A was then dialysed in phosphate buffer pH 5.0 containing 2-fold molar excess of copper. Spectra were recorded after overnight incubation of 50 μM AcoP M171A against an excess of acetate, ethanol, azide, thiocyanate, imidazole or DMS. In some cases, some scattering could be observed around 350 nm and might be due to small aggregates arising from the chemical treatment.

Cu-S(Cys) bond. These changes are elicited by a rotation of the (Cys)S-Cu-S(Met) plane with respect to the (His)N-Cu-N(His) plane, introducing a tetragonal distortion [16,20]. Future structural analysis is required in order to understand if the same determinants are responsible for the green copper center geometry in AcoP.

Table 5
Experimental UV–Vis ABS spectroscopic parameters of AcoP M171A after the incorporation of various axial exogenous ligands.

	λ_1	λ_2	R_L
Phosphate	395 nm	500 nm	2.42
+ ethanol	390 nm	498 nm	2.8
+ acetate	368 nm	455 nm	6.25
+ azide	374 nm	458 nm	2.0
+ thiocyanate	378 nm	485 nm	2.0
+ imidazole	410 nm	500 nm	2.1
+ dimethylsulfide	378 nm	495 nm	2.

Our results demonstrate that no spectroscopic features could be measured when either of the two histidines was mutated. Even the addition of exogenous ligands like imidazole did not allow the reconstitution of a redox responsive copper site, meaning that no accessible space has been made by mutating AcoP His85 and His166 into alanine. By comparison with previous spectroscopic, redox and structural studies of T1 copper centers [43,46–47], we propose that AcoP histidine mutants engender a tricoordinated copper site, the destabilization of the Cu^{2+} state, and thus a loss of redox features and of measurable UV–Vis ABS and EPR spectra.

Replacing the axial methionine by an alanine has little effect on the geometry of classic T1copper sites. This is the case for the M98A mutant of amicyanin, the M144A mutant of the blue Nir from Alcaligenes xylosoxidans and the M121A azurine mutant [28, 54,57]. However, by adding exogenous ligands that bind strongly to the copper, like imidazole that mimics His, a blue copper center can then be modified towards a green or a red geometry [56,58]. In AcoP M171A, depletion of the axial methionine results in drastic changes in the copper center geometry and redox properties, suggesting the presence of a stronger binding of the axial Met to the copper ion, in line with the observations of other green copper sites. Removal of the axial Met side-chain allows binding of several exogenous ligands that impact on the metal site structure, but do not restore the wild type green nor a classic blue site. Without the methionine side chain, the copper center tends towards a more square planar type. Our results suggest that the position and rigidity of the axial ligand, and not only its chemical nature, may play a key role in favoring AcoP copper center green geometry.

Mutating histidine or methionine ligands affects not only copper geometry, but also redox properties of the metal center. Indeed, none of the mutant neither the reconstituted AcoP mutant copper sites is redox active, meaning that copper is stabilized in either its cuprous or cupric state. Such drastic change of the redox potential was unexpected because usually cupredoxins axial ligands are known to mainly fine tune the redox potential [27,34]. The behaviour of several natural cupredoxins seems to fit into a model where shorter (Cys)S-Cu and longer (Met)S-Cu bonds correlate with higher redox potential, as seen in blue cupredoxins, whereas longer (Cys)S-Cu and shorter (Met)S-Cu bonds correlate with lower redox potential, as seen in the green NiR [18]. Indeed, the mutation of the methionine axial residue of the green NiR of R. sphaeroides to threonine, a weak axial interaction leads to an increase of redox potential from 247 mV to 350 mV [33]. The same trend is observed for Auracyanin D, for which a strong and close axial ligand (glutamine) and low redox potential are observed (90 mV at pH 7 vs SHE) [36]. Mutation of its axial ligand to leucine drastically increases the redox potential (786 mV at pH 7 vs SHE) [37]. In line with this, addition of exogenous strong ligands or the introduction of a strong axial ligand like glutamine lowers the redox potential [13,18,59] On the contrary, in proteins like MCO, a non-coordinating axial leucine results in the highest redox potentials known to date for a cupredoxin fold and the introduction of weak ligands either by site directed mutagenesis or exogenously leads to higher redox potential [13,18] Based on these data, it has been proposed that a shorter (Met)S-Cu^{2+} distance may result in higher stabilization of the Cu^{2+} state, leading to lower redox potential [13,19,25]. Intriguingly, Nir and Auracyanin D have a low redox potential and AcoP an unusually high one eventhough they share a similar distorted green geometry. The determinants of high redox potential in AcoP need to be unveiled.

Finally, here we have identified the copper ligands in AcoP and assessed their role in copper ligation, that resembles that of other green sites. However, it is still intriguing whether the ligand set itself gives rise to this high redox potential. Our data suggest that the protein scaffold plays a crucial role in determining the intrinsic properties of the cupredoxin AcoP. Further studies in this system will be required to dissect the individual contributions to its electronic and redox features.

4. Methods

4.1. Protein mutagenesis, expression and protein purification

QuikChange site-directed mutagenesis Kit (Stratagene) was used to generate the H85A, C159A, H166A, M171A, H172A, and M171A-H172A mutants. DNA sequences used to construct site-directed mutants were designed using the QuikChange Primer Design program, and oligonucleotides synthesized by Invitrogen. Mutants were generated by PCR amplification of the Gateway™ pDEST17 plasmid in which the acoP gene from A. ferrooxidans ATCC 23270 had been previously subcloned. Sequencing by Cogenics confirmed mutant identity, and bacterial cells from the Escherichia coli BL21 (DE3) expression strain were transformed with these plasmids.

Protein expression and purification were performed as described previously [40]. The quantity of purified apo-AcoP was determined by UV–Vis ABS spectroscopy by measuring the absorption intensity at 280 nm and using a sequence-derived theoretical molar extinction coefficient (ε_{280}) of 25,440 M^{-1} cm^{-1} to estimate protein concentration. Purified holo-AcoP were obtained by incubation of the apo form with two fold-molar excess of copper at pH 5.0 in 50 mM sodium acetate buffer containing 0.005%(w/v) n-dodecyl β-D-maltoside (DDM) and 5%(v/v) glycerol. The excess of copper was removed using a gel filtration column (PD10, Pharmacia) pre-equilibrated in the same buffer. When mentioned, purified holo-AcoP were dialyzed during at least 3 h against a 20 mM sodium phosphate or sodium citrate buffer buffer containing 0.005%(w/v) DDM and 5%(v/v) glycerol for which the pH was adjusted to 2.5, 5.0 or 7.5.

4.2. Spectroscopic characterization

For UV–Vis ABS measurements, protein concentration was typically at 50 μM. Room temperature UV–Vis ABS spectra were recorded on a Cary 50 Bio (Varian) spectrophotometer. Low Temperature spectra were obtained with a Cary 300 (Varian) spectrophotometer modified to accommodate a Dewar mounted in the light path. This arrangement allows data collection at around 77 K. For this experiment, samples were diluted in buffer 50 mM sodium acetate containing 70%(v/v) glycerol. R_L values correspond to the ratio between ~440 and ~600 nm peaks for green or blue perturbed copper sites or between ~368 and ~455 nm for red copper sites [11].

Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter at 298 K in a 1 mm path length cell. 20 μM of proteins in 50 mM sodium acetate at pH 5.0 was used. Spectra were averaged from five scans, and normalized for any variation in protein concentrations. Vis CD spectra were recorded in the region of 350 to 750 nm with 50 μM of holoproteins. Both UV–Vis ABS and Vis CD spectra were overlaid to cover the entire energy region from 750 to 350 nm, and fit to Gaussian line shapes using PeakFit 4.12 from Jandel.

Elemental analyses of copper were performed using an iCAP-7000 ICP-OES analyzer (Thermo Scientific) on 50 μg of purified proteins.

EPR spectra were collected at X-band (9.4 GHz) on a Bruker ELEXSYS E500 spectrometer equipped with an ER4102ST standard rectangular Bruker EPR cavity fitted to an Oxford Instruments ESR 900 helium flow cryostat.

4.3. Cyclic voltammetry

Cyclic voltammetry (CV) was carried out using Autolab potentiostat. In a typical experiment, 2 μl of 50 μM protein in 10 mM sodium acetate buffer pH 5.0 was entrapped between the surface of a PG electrode and a dialysis membrane of appropriate cutoff. This design allows to work under thin layer configuration [42]. CV scan rate was typically 10 $mV.s^{-1}$.

4.4. Exogenous ligand incorporation

Complexes of AcoP M171A with exogenous molecules were obtained using the following procedure. HoloAcoP M171A (50 μM in 50 mM sodium acetate buffer pH 5.0, 0.005% DDM and 5% glycerol) was dialysed against 20 mM sodium phosphate-citrate buffer pH 2.5, 0.005%DDM and 5%glycerol, in order to destabilize the binding of copper. This step was followed by a second dialysis in a 20 mM phosphate buffer at pH 5.0, 0.005%DDM and 5%glycerol. AcoP M171A was subsequently incubated with a large excess of exogenous ligand (sodium acetate, sodium azide, sodium thiocyanate, imidazole, ethanol, DMS) at 4 °C overnight.

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