Cupredoxins are type 1 copper center-containing electron transfer proteins involved in many biological processes, such as photosynthesis, respiration and cell signaling.\cite{1} In addition to small electron transfer proteins, the type 1 copper (T1C) center also appears in larger proteins, such as copper-containing nitrite reductase (CuNiR) and laccase. The T1C center has unique spectroscopic characteristics, including intense electronic absorption ($\sim 5000 \text{ M}^{-1} \text{ cm}^{-1}$) at around 600 nm and small hyperfine splitting ($\approx 100 \times 10^{-4} \text{ cm}^{-1}$) in the electron paramagnetic resonance (EPR) spectrum, which is attributed to its coordination environment.\cite{2,3} Cupredoxins and protein domains in larger proteins with a T1C center share great structural similarity: within a common cupredoxin fold, the copper center is located at one end of the protein, and the metal-binding loop connecting the 6th and 7th $\beta$ strand harbors 3 copper-binding ligands (Fig. 1 and 2A).\cite{3} In cupredoxins and related red copper protein and CuA proteins, the metal-binding loop varies in length and sequence (Fig. 1A–G). For cupredoxins and protein domains with a T1C center, the loop sequences are in the general formula of $\text{C}x\text{H}_x\text{M}$, where $X$ denotes any amino acid and $m$ and $n$ are the number of residues in between, $C$, $H$ are conserved copper ligands and $M$ is methionine or another axial residue. Values of $m$ and $n$ are 2–4 for native proteins with T1C center and the value of $m$ could be as large as 8 in domains containing the T1C center, such as in CuNiR.\cite{4}

Due to its importance, the metal-binding loop has been the target for engineering. Two groups have independently replaced the metal-binding loop of cupredoxins with the same region of CuNiR domain of Cytochrome c Oxidase (CcO), converting the protein with a T1C center into a protein with Cu$_2$ center, a binuclear copper center (Fig. 2G).\cite{5,6} Berry and coworkers have converted the type 1 blue copper center to a red copper center, by replacing the metal-binding loop of azurin (Az) from that of nitrosocyanin.\cite{7} The metal-binding loop not only influences the electronic structure of the protein, but also affects the reduction potential. Canters, Dennison and others have replaced the metal-binding loop of one cupredoxin with that of another one.\cite{8} The hybrid protein maintains T1C characters, and its reduction potential shifts toward that of the loop donor.

Given the diversity of the metal-binding loop of cupredoxins (Fig. 1), researchers have been exploring what sequences can be tolerated in the metal-binding loop, and how they influence the reduction potential of the protein, using Az and other cupredoxins as a model. Dennison and coworkers have shortened the loop of Az from C$_{112}$TPGH$_{117}$SALM$_{21}$ to C$_{112}$TPH$_{117}$PM$_{18}$ and C$_{112}$TPH$_{117}$PM$_{17}$, while keeping the mutants T1C characteristics.\cite{9} The CTPHPM loop, with $m = 2$ and $n = 1$, is the shortest loop presented in a cupredoxin. The same group has connected the copper ligands with different numbers of Ala (Fig. 2H).\cite{10} The loop are able to accommodate 2–4 amino acids between copper ligands, while keeping type 1 copper characteristics. As shown in the same work, when only one Ala are present between Cys112, His117 and His117, Met121, the protein does not bind Cu. Changing the 15-residue loop of CuNiR to that of amicyanin (7 residues) resulted in a 200 mV increase of reduction potential of the T1C center, abolishing NiR's enzymatic activity.\cite{11} On the other hand, amicyanin can
accommodate ligand loop of CuNiR, which has 8 residues between Cys and His and 4 residues between His and Met. It is not clear the upper limit of number of residues in the loop. Here, we tested an extreme case, where through circular permutation, Phe114 and Gly116 becomes the first and last residue of the new protein, essentially making the number of residues between Cys112 and His117 approaching infinity (Fig. 1I and 2B). In a previous study, two construct have been built for circular permutation in Az. The mutants, cpAz1 and cpAz2, maintain typical TIC spectroscopic properties, suggesting the robustness of Az structure. However, as the metal-binding loop harbors 3 ligands of copper center, the question remains whether circular permutation will perturb the copper center. Here we report the design, spectroscopic and structural characterization of the new protein, termed Az-loop.

Materials and methods

Vector construction

The following primers were used to amplify 346–381 and 1–342 of WT Az gene, with a common linker sequence ggaattccgggtggc.

NcoI-116-f:
CAGCCGGCGATGGCCatgggtcactccgcactgatgaaaggtacc

127-linker-r: gccacccggaattcctttcagagtcagggtacctttcatcagtgcg

linker-1-f: gaaaggaattccgggtggcgctgaatgctccgttgatatccag

114-NotI-r:
GTGCTCGAGTGCGGCCGCTcagaaagtgcagaagaacatgtactgttc

The two fragment were used as template to amplify the coding sequence of Az-loop. The linear fragment was digested with NcoI and NotI and cloned into pET22 vector.

Nucleotide sequence of Az-loop:
atgggtcactccgcactgatgaaaggtaccctgactctgaaaggaattccgggtgg
cgctgaatgctccgttgatatccagggt aatgatcagatgcagttcaacaccaacgcca
tcaccgtcgacaagagctgc aagcagttcactgttaacctgtctcacccaggtaacctgc
cgaagaacgttatgggtcac aactgggttctgtccaccgcggctgacatgcaaggcgt
tgcactgacggtatggctagcggtctggataaagactacctgaagccggatgactctcga
gttatcgcccacaccaagctgatcggatccggtgaaaaagactccgttactttgacgtttc
cagcttaaagaaggtgaacagtacatgttcttctgcactttctga

Protein purification and crystallization

Following the previously reported procedure for Az purification, the protein was further purified using size-exclusion chromatography (Superdex G-75 10/300 GL, GE Healthcare).
Absorption spectrum of Az-loop was recorded on a MAPADA UV3100 spectrometer. Amount of copper ion in the protein was analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) on a PerkinElmer NexION 300X spectrometer, calibrated by standard solutions from Sigma. Extinction coefficient of copper was calculated based on the electronic absorption spectrum and copper concentration of the same sample.

X-band EPR spectra were collected on a JEOL JES-FA200 spectrometer at the Analysis Center of Tsinghua University. 1.2 equivalence of Cu(II) ion were added into apo WT Az and Az-loop. Excess Cu(II) ion were removed by a short Sephadex G-25 column. The proteins were further purified by anion exchange (Hitrap Q HP, GE Healthcare) and size exclusion (Superdex G-75 10/300 GL, GE Healthcare) chromatography. The samples containing 3–4 mM Cu(II) and 20% glycerol were run at ~123 K using liquid nitrogen. EPR spectra were simulated with hyperfine spectrum.29

**Data collection and structure determination**

Diffraction data were collected at BL17U of the Shanghai Synchrotron Radiation Facility (SSRF).30 HKL2000 was used for data processing.31 The structure was solved by molecular replacement using Phaser in CCP4 (ref. 32 and 33) with WT Az structure (PDB ID: 4AZU) as the searching model. Coot and Refmac5 were used for model building and refinement.44,35

**Electrochemical characterization**

The reduction potential of Az-loop was determined by cyclic voltammetry after verifying the WT Az reduction potential on CH Instruments 620A potentiostat. Briefly, 10 μL of carbon nanotube (2 mg mL⁻¹) dispersed in ethanol was applied on the surface of glassy carbon electrode and let dry.45 2 μL of Az (1.5 mM) was applied on top of the carbon-nanotube paste. After a short incubation time, the electrode was immersed in either 50 mM NH₄OAc, pH 4.0 with 100 mM NaCl, or 50 mM NH₄OAc, pH 7.0 with 100 mM NaCl before data collection. The reduction potentials were measured against Ag/AgCl and converted to those against NHE by adding 210 mV to the obtained value calculated based on the value of ferricyanide control.

**Results and discussion**

**Design of circularly permutated Az variant**

As shown in Fig. 2B, here we applied circular permutation strategy, to connect N- and C-termini with a linker GIPGG and create new termini at Gly116 (N-terminus) and Phe114 (C terminus), similar to what have been reported for cpAz1 and cpAz2.27

The Az-loop mutant was constructed by overlap-extension PCR. The protein was purified in a procedure similar to that of the WT protein28 to obtain metal-free apo protein with a yield of ~40 mg L⁻¹. The identities of the Az-loop protein was verified by electrospray mass spectrometry after purification (Fig. S1†).

**Spectroscopic and electrochemical characterization of Az-loop**

Addition of Cu²⁺ into the apo protein gave the protein blue color. Absorption spectrum of the protein showed a λmax of 624 nm, only slightly blue-shifted from that of WT Az (627 nm). The absorption at 624 nm is assigned as p_{(Cys)}–Cu(II) charge transfer band (Fig. 3A). As the hallmark of cupredoxins, the
intense absorption indicates that the mutant protein has an unperturbed TIC center.

EPR spectroscopy provides a more sensitive way to characterize copper coordination environment. As shown in Fig. 3B, EPR spectrum of Az-loop showed more than one species. Simulation of the spectrum showed the majority of the signal (84%) is from a type 1 center with the same parameters as WT Az, while the rest of the signal is from a type 2 center with large hyperfine splitting values ($200 \times 10^{-4}$ cm$^{-1}$, Fig. S2 and Table S1†). Addition of equal amount of apo WT Az into Cu$^{2+}$-loaded Az-loop did not cause significant change in electronic absorption spectrum (Fig. S3†), suggesting that species 2 is not adventitious Cu(n) in the sample. Since species 2 is likely a type 2 center, its absorption at 600 nm region is weak, which accounts for the lower than WT extinction coefficient. Due to its low abundance, no additional feature is observe in electronic absorption spectrum.

The redox potential of Az-loop was measured by cyclic voltammetry using a carbon nanotube modified glassy carbon electrode. Redox potential Az-loop was determined to be $353 \pm 2$ mV and $239 \pm 12$ mV at pH 4 and pH 7, respectively, which is 22 and 13 mV higher than that of the WT (Fig. S4†). The potential can be assigned to the species with a type 1 center due to its high abundance and similarity to the WT protein.

In the loop transfer mutants, where loop of a cupredoxin is transferred to another cupredoxin, the spectroscopic properties and reduction potentials of the protein are dictated by the loop donor.24 When the residues between copper ligands in the loop are changed to the same Ala/Gly/Val (Fig. 2H), the copper center in Az still forms.25 As Dennison and coworkers suggested, the reduction potential of the mutants is not affected by the length of the loop, but by the polarity of the copper binding site. In Az-loop, although the length of the loop is infinity due to circular permutation, hydrogen bonding and hydrophobicity of the site does not change since the native residues are unchanged. As expected, the reduction potential of Az-loop is similar to that of WT Az.

The $p_{K_a}$ values of C-terminal His in reduced cupredoxins range from 4.3 to 6.7 for most cupredoxins, including amicyanin, plastocyanin, pseudoazurin, stellacyanin, and cucumber basic protein,4 while the value for Az is estimated to be below 2.18 The length of the metal-binding loop has long been proposed to affect the $p_{K_a}$ value.39 Dennison and coworkers have found that in Az mutants with engineered loop, the $p_{K_a}$ values of C-terminal His increased to 4.4 or higher.25,26,40 A $\Delta - 60$ mV pH$^{-1}$ change of reduction potential in response to pH between pH 4–7 was observed in the mutants with higher $p_{K_a}$, while in WT Az the reduction potential is not affected by pH below pH 5.5.25 In Az-loop, the difference of reduction potential between pH 4 and pH 7 is 112 mV, similar to that of the WT Az, suggesting the $p_{K_a}$ value of His3 (corresponding to the C-terminal His117 in WT Az) is smaller than 4.

**Structural characterization**

To investigate the effect of circular permutation on the copper coordination, as well as on the overall structure of the protein, we crystallized Az-loop and the crystal was diffracted to 1.66 Å resolution. The structure of Az-loop was refined to an $R_{free}$ of 0.217 (Fig. 4 and Table S2†). Coordinates and structure factors were deposited in the Protein Data Bank (accession code: 5YT7).

As shown in Fig. 4A, structure of Az-loop overlaid well with that of WT Az with a RMSD of backbone atoms of 0.74 Å. The GIPGG linker forms a loop, connecting the original N- and C-termini. In the metal-binding loop, although the constraint of circular permutation on the copper coordination environment is expected to be relatively small, the loop still follows its original direction.

The distances between copper and its ligands are listed in Table S3†. Cys-Cu distance is 0.06 Å and Met-Cu distance is 0.11 Å longer than these in the WT structure (PDB ID: 4AZU). These distances are within the normal range observed in cupredoxins.3 The un-perturbed primary ligands leads to the intense 624 nm absorbance of the protein. The structure likely represents species 1 with a type 1 copper center observed...
in EPR spectrum. Beyond copper ligands, residues interact with copper ligands are also important for reduction potential of Az.\textsuperscript{6,41} We looked into the distances in the second coordination sphere. Only distance between N6 of N66 and S\textgamma of Cys131 changes more than 0.05 Å, compared to that of the WT protein. The change alone is not sufficient to alter the reduction potential significantly. Previously observed spectroscopic and electrochemical properties of Az-loop can be explained by its conserved primary and secondary coordination sphere structure.

In WT Az, the copper ion is shielded by its ligands and not accessible by solvent or external ligands. Mutating His or Met to smaller residues increases solvent accessibility of the site.\textsuperscript{1,30}\textsuperscript{30} Equivalence of histidine or N3\textsuperscript{30} strong ligands known to cause absorption spectrum change, was added into Az-loop (Fig. S5f). The absorption spectrum of Az-loop remain unchanged. The surf rendering of WT Az and Az-loop showed similar structures (Fig. S6f). There is no indication of change in solvent accessibility in Az-loop.

Fig. 4 Crystal structure of Az-loop. Overlay of crystal structure of WT Az (yellow, PDB: 4AZU) and Az-loop (cyan, PDB: 5YT7). Active site residues were in licorice style and nearly overlaid. The metal-binding loop in WT Az and the loop connecting the original N- and C-termini in Az-loop were highlighted in red. Comparison of copper center of WT Az (B) and Az-loop (C).

Besides EPR spectroscopy, species 2-the one with type 2 copper center remains silent in all other characterizations. In addition to the fact that it is a minor species, weak electronic absorption and low electron transfer activity associate with type 2 sites may also contribute to its reclusive nature. Interestingly, EPR fitting of species 2 showed a larger gX and smaller gZ than typical type 2 sites. As a result, it is hard to determine the exact structure of the species.

**Conclusion**

In the work, we reported construction of an Az mutant of infinite metal-binding loop length by circular permutation. The mutant, Az-loop, exhibited absorption spectrum typical of T1C proteins. EPR spectrum of Az-loop showed a mixture of two species, and the major species contains a type 1 copper center. The reduction potential of Az-loop is close to that of WT Az, indicating circular permutation has minimal perturbation to the structure. The minimal structure perturbation is further confirmed by structure analysis of the mutant Az-loop, which indicates the overall structure and primary and secondary coordination sphere of Az-loop are similar to that of the WT protein.

The finding that circular permutation does not alter the protein is consistent with Dennison and coworker’s finding, which suggests that the metal-binding loop is flexible enough to tolerate different sequences and length while maintaining protein’s T1C characters.\textsuperscript{24,47} The flexibility is important for metallation of cupredoxins. Lu and coworkers and Blackburn and coworkers have reported intermediates during CuA center formation in an engineered CuA-Az and CuA domain of *T. thermophilus* Cytochrome c Oxidase, suggesting ligand rearrangement and loop movement in this process.\textsuperscript{42,43} Such arrangement is impossible in a rigid structure. Vila and coworker have determined NMR structure of Az and CuA domain of *T. thermophilus* CcO, a closely related protein with CuA center, further confirming the flexibility of the loop.\textsuperscript{44}

As a method for protein engineering, circular permutation has been applied in constructing different sensors.\textsuperscript{45,46} Our work opens the possibility to attach a redox active protein or small molecule at the termini of Az while keeping the redox centers close for electron transfer.

**Conflicts of interest**

There are no conflicts to declare.

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**References**
