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The sole tryptophan of amicyanin enhances its thermal stability but does not influence the electronic properties of the type 1 copper site ☆

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ABSTRACT

The cupredoxin amicyanin possesses a single tryptophan residue, Trp45. Its fluorescence is quenched when copper is bound even though it is separated by 10.1 Å. Mutation of Trp45 to Ala, Phe, Leu and Lys resulted in undetectable protein expression. A W45Y amicyanin variant was isolated. The W45Y mutation did not alter the spectroscopic properties or intrinsic redox potential of amicyanin, but increased the pK_a value for the pH-dependent redox potential by 0.5 units. This is due to a hydrogen-bond involving the His95 copper ligand which is present in reduced W45Y amicyanin but not in native amicyanin. The W45Y mutation significantly decreased the thermal stability of amicyanin, as determined by changes in the visible absorbance of oxidized amicyanin and in the circular dichroism spectra for oxidized, reduced and apo forms of amicyanin. Comparison of the crystal structures suggests that the decreased stability of W45Y amicyanin may be attributed to the loss of a strong interior hydrogen bond between Trp45 and Tyr90 in native amicyanin which links two of the β-sheets that comprise the overall structure of amicyanin. Thus, Trp45 is critical for stabilizing the structure of amicyanin but it does not influence the electronic properties of the copper which quenches its fluorescence.

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Introduction

Type 1 copper sites are found in a wide range of redox proteins in bacteria, plants and animals, and function as electron transfer mediators [1,2]. In the type 1 site a single copper is coordinated by three equatorial ligands that are provided by a Cys and two His residues, and by a fourth weak axial ligand, usually provided by a Met. These ligands hold the copper in a distorted tetrahedral geometry. Cupredoxins are small soluble type 1 copper proteins with a single copper site which are characterized by an intense blue color and absorption centered near 600 nm that results from a S(Cys)π → Cu(II)d_{x2-y2} ligand-to-metal charge transfer transition [3]. Amicyanin from *Paracoccus denitrificans* [4,5] is a cupredoxin which mediates electron transfer from methylamine dehydrogenase (MADH)[1] [6] to cytochrome *c*-551i [7]. Crystal structures of amicyanin alone [8] and in complex with its redox partner proteins [9,10] have been determined, and spectroscopic, redox, kinetic and site-directed mutagenesis studies have described structure–function relationships that define the roles of specific amino acid residues of amicyanin in recognition of redox partners and mediation of interprotein electron transfer [11–17].

An interesting feature of *P. denitrificans* amicyanin is that it contains a single tryptophan residue, Trp45 which resides in the hydrophobic core of the protein. While Trp45 is 10.1 Å from the copper, the intrinsic fluorescence from this tryptophan is quenched when copper is bound relative to the fluorescence that is observed with apoamicyanin from which copper has been removed [18]. The

☆ Coordinates and structure factors have been deposited as Protein Data Bank entries 4P5R (oxidized W45Y amicyanin) and 4P5S (reduced W45Y amicyanin).

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[1] *Abbreviations used:* CD, circular dichroism; E_m, oxidation–reduction midpoint potential; MADH, methylamine dehydrogenase; rms, root mean square; T_m, midpoint temperature for a spectroscopic transition; WT, wild-type.

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cupredoxins azurin [19] and stellacyanin [20] also possess a tryptophan residue located within 10 Å of the copper ion and each exhibits the phenomenon of tryptophan fluorescence quenching upon metal ion binding [21,22]. This metal dependent fluorescence quenching has been attributed to either Forster energy transfer or electron transfer [23–25]. In azurin, a structurally analogous Trp residue has been shown to participate in hopping-mediated electron transfer between a rhenium ion attached to the protein surface and the copper of the type 1 site [26]. The finding that the copper of the type 1 site in amicyanin influences the electronic properties of Trp45, and the evidence that this Trp could participate in hopping-mediated electron transfer, raise the question of whether or not the presence of tryptophan in this position influences the properties of the type 1 copper site of amicyanin.

It has been suggested that this tryptophan in cupredoxins is part of an interior core of hydrophobic residue pairs. The spatial orientation of the hydrophobic residue pairs is conserved in azurin, amicyanin, rusticyanin, nitrocyanin, pseudoazurin, plastocyanin, stellacyanin, and auracyanin [27]. The roles of several of these hydrophobic residues in the protein folding behavior of azurin from *Pseudomonas aeruginosa* was studied by site-directed mutagenesis, however Trp was not one of the residues that was altered [27]. Thus, another question regarding Trp45 of amicyanin is whether this residue is an important determinant of the overall structure and stability of this type 1 copper protein.

To address these questions, Trp45 was altered by site-directed mutagenesis and the effects of this change on the structure and function of amicyanin were assessed. W45A, W45F, W45L and W45K mutations resulted in no detectable protein from the expression system. Only a W45Y mutation was tolerated by the protein. The effects of this mutation on the structure of the protein, the spectroscopic and redox properties of copper site, the affinity of the protein for copper, and the thermal stability of the protein were determined.

Materials and methods

Protein expression and purification

Methods for the expression and purification of MADH [28], cytochrome *c*-551i [7] and wild-type (WT) amicyanin [4] from *P. denitrificans* were as described previously. The Phusion site-directed mutagenesis kit was used to create W45A, W45F, W45L, W45K, and W45Y amicyanin variants. The forward primers for Trp45 mutations (mutated nucleotides are underlined) were W45A, 5′-CGTCACCGCGATCAAC-3′; W45F, 5′-CGTCACCTTCATC AACCG-3′; W45Y, 5′-CGTCACCTACATCAACCGC-3′; W45L, 5′-CGT CACCCTAATCAACCG-3′; W45K, 5′-CGTCACCAAGATCAACCG-3′. In each case the reverse primer was 5′-GTGTCGCCGACCTTCAC-3′. Mutagenesis was performed using pMEG201 [12] which contains the *mauC* gene [29] which encodes amicyanin. The entire 555-base *mauC*-containing fragment was sequenced to ensure that no second site mutations were present, and in each case none were found. Amicyanin variant proteins were expressed in *Escherichia coli* BL21 cells and isolated from the periplasmic fraction as described for other recombinant amicyanin variants [12].

Reduced amicyanin was prepared by titration with sodium dithionite until absorbance at 595 nm was completely lost. Apoamicyanin was prepared as previously described [18] by dialyzing reduced amicyanin against 0.1 M potassium cyanide in 0.1 M Tris–HCl buffer (pH 8.0) for 20 h, followed by dialysis against 10 mM potassium phosphate buffer (pH 7.1) for 4 h.

Protein crystallization and X-ray structure determination

W45Y amicyanin was washed with 5 mM Na/K phosphate buffer, pH 6.6 before the crystallization trials following the protocol used previously for WT amicyanin and some amicyanin variants [5,30,31]. The Hampton Research's ammonium sulfate screen (HR2-211) was used to get initial crystals. These crystals were used as seeds to grow crystals suitable for X-ray data collection using 90:10 mixture of 3.2 M monobasic sodium:dibasic potassium phosphate solution. The reduced form of W45Y amicyanin was produced by soaking the crystals in an artificial reservoir solution containing 80 mM sodium ascorbate in the same buffer solution for 20 min. Both the oxidized and reduced crystals were cryo-protected with krytox oil for data collection.

X-ray data from crystals of oxidized and reduced W45Y amicyanin were recorded at the 24lD-E and 24lD-C beamlines of NECAT, Advanced Photon Source (APS), equipped with Microdiffractometer-MD2 and ADSC Quantum 315 CCD detector (24lDE)/Pilastus6MF (24lDC). These data were processed using DENZO and scalepack, as a part of the HKL2000 package [32]. A fluorescence scan was carried out at the Cu absorption energy at 24lDC, NECAT, APS to confirm the presence of Cu ion in both oxidized and reduced forms.

The structures of oxidized and reduced W45Y amicyanin were solved by molecular replacement method using PHASER [33] of PHENIX [34] using the coordinates for native amicyanin (PDB entry lAAC) with Trp45 mutated to Ala. There was a single molecule in the asymmetric unit. Based on difference Fourier electron density maps using COOT [35], Ala was changed to Tyr45. To monitor the refinement, a random subset of all reflections (5%; 1661 reflections for oxidized state and 1985 reflections for reduced state) was set aside for R_{free} calculation [36]. An anomalous difference Fourier map was computed for both oxidized and reduced forms to confirm the presence of copper. The refinement of W45Y amicyanin was carried out using PHENIX by subjecting the model to alternative positional and B-factor refinement. A simulated annealing refinement was performed at the beginning of the refinement. No restraints were applied to the metal, ligand distances or bond angles. A total of 171 water molecules were added to both the oxidized and reduced W45Y model. The final R/R_{free} values of the model are 12.7/15.4 for oxidized and 13.4/15.0 for reduced forms, respectively. The final models contain one copper and one sodium ion for the oxidized form and only copper ion for reduced form. For the oxidized form, the average temperature factor is 8.2 Å^2 for all protein atoms, 15.6 Å^2 for water molecules and 5.2 Å^2/10.4 Å^2 for copper/sodium ions. For the reduced form, the average temperature factor is 8.8 Å^2 for all protein atoms, 19.4 Å^2 for water molecules and 7 Å^2 for copper ion. A significant negative density along with positive density observed around the copper position in the reduced form (Fig. 2C). The Ramachandran map calculated for both oxidized and reduced forms using PROCHECK [37] show that all the non-glycine residues are either in most favored or in additional allowed regions. The rms deviation calculation and structure analysis were carried out using the programs COOT [35], CCP4MG [38] and CCP4 [39].

Resonance Raman spectroscopy

Resonance Raman spectra of 2 mM WT and W45Y amicyanin were measured with a micro-Raman system (Horiba Jobin Yvon, LabRam HR) at an excitation wavelength of 632.8 nm. Spectra were recorded with a back-thinned CCD detector at a spectra resolution of 2 cm^{-1}. Calibration was performed with silicon and naphthalene standards.

Redox potential determinations

Oxidation–reduction midpoint potential (E_m) values were determined by spectrochemical titration as described previously for amicyanin [40]. The ambient potential was measured directly with a redox electrode which was calibrated using quinhydrone

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