Published in final edited form as:

Arch Biochem Biophys. 2014 May 15; 0: 20–27. doi:10.1016/j.abb.2014.03.010.

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

Brian A. Dow¹, Narayanasami Sukumar^{2,*}, Jason O. Matos^{1,3}, Moonsung Choi⁴, Alfons Schulte³, Suren A. Tatulian³, and Victor L. Davidson^{1,*}

¹Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32827

²NE-CAT and Department of Chemistry and Chemical Biology, Cornell University, Building 436E, Argonne National Laboratory, Argonne, IL, 60439

³Department of Physics, University of Central Florida, Orlando, FL 32816

⁴Seoul National University of Science and Technology, College of Energy and Biotechnology, Department of Optometry, Seoul, 139-743, Korea

Abstract

The cupredoxin amicyanin possesses a single tryptophan residue, Trp45. Its fluorescence is quenched when copper is bound even though it 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.

© 2014 Elsevier Inc. All rights reserved.

*Corresponding authors: Victor L. Davidson, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd., Orlando, FL 32827 Tel: 407-266-7111. Fax: 407-266-7002. victor.davidson@ucf.edu; Narayanasami Sukumar, Building 436E, APS, Argonne National Laboratory, 9700 S. Cass Ave., Argonne, IL 60439. Tel: 630-252-0681. sukumar@anl.gov.

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

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

amicyanin; cupredoxin; hydrogen bond; protein structure

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 $S(Cys)\pi \rightarrow Cu(II)d_{x^2-y^2}$ ligand-to-metal charge transfer transition [3]. Amicyanin from *Paracoccus denitrificans* [4, 5] is a cupredoxin which mediates electron transfer from methylamine dehydrogenase (MADH) [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 *Paracoccus 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 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'-CGTCACCTTCATCAACCG-3'; W45Y, 5'-CGTCACCTACATCAACCGC-3'; W45L, 5'-CGTCACCCTAATCAACCG-3'; W45K, 5'-CGTCACCAAGATCAACCG-3'. In each case the reverse primer was 5'-GTGTGCGCCGACCTTCAC-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 *E. 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 hr, followed by dialysis against 10 mM potassium phosphate buffer (pH 7.1) for four hr.

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 24ID-E and 24ID-C beamlines of NECAT, Advanced Photon Source (APS), equipped with Microdiffractometer-MD2 and ADSC Quantum 315 CCD detector (24IDE)/Pilatus6MF (24IDC). 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 241DC, 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 1AAC) 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 174 and 171 water molecules were added to 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 $8.2 \text{ \AA}^2$ for all protein atoms, $15.6 \text{ \AA}^2$ for water molecules and $5.2 \text{ \AA}^2/10.4 \text{ \AA}^2$ for copper/sodium ions. For the reduced form, the average temperature factor is $8.8 \text{ \AA}^2$ for all protein atoms, $19.4 \text{ \AA}^2$ for water molecules and $7 \text{ \AA}^2$ for copper ion. A significant negative density along with positive density observed around the copper position in the reduced form (Figure 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 (a 1:1 mixture of hydroquinone and benzoquinone) as a standard with an E_m value of 286 mV at pH 7.0 [41]. The titrations were performed in 10 mM potassium phosphate buffer at the indicated pH, at 25 °C. The mixture was titrated by addition of incremental amounts of a reductant, ascorbate, or an oxidant, ferricyanide. The concentrations of oxidized and reduced amicyanin were determined by comparison with the spectra of the completely oxidized and reduced forms. The data were analyzed according to eq 1 to determine the E_m value. The pH-dependence of the E_m value was determined by eq 2, where $E_{m\text{Alk}}$ is the most alkaline E_m measured that is in the pH-independent region [40], R is the gas constant, T is

temperature (K), n is the number of electrons, K_a is the acid dissociation constant and F is Faraday's constant.

$$E = E_m + (2.3RT/nF) \log([Amicyanin_{oxidized}]/[Amicyanin_{reduced}]) \quad [1]$$

$$E_m = E_{mAlk} + (2.303RT/nF) \log(1 + ([H^+]/K_a)) \quad [2]$$

Kinetic studies

The steady-state kinetic parameters for amicyanin with its natural redox partner proteins were determined as previously described for two different reactions. One reaction is the methylamine-dependent reduction of amicyanin by MADH [42]. The other is the methylamine-dependent reduction of cytochrome *c*-551 by the MADH-amicyanin complex [43]. These reactions were performed in 10 mM potassium phosphate pH 7.5 buffer at 30 °C.

Temperature dependence of amicyanin absorbance (optical melt)

Absorbance measurements of oxidized amicyanin were made with an HP diode array 8452 spectrophotometer equipped with a programmable Peltier cell holder controlled by a Quantum TC1 thermostat controller. Absorbance measurements at 595 nm were recorded as the cell increased in temperature 1°C/min. Absorbance values were normalized with the maximum absorbance set to 1.0 and the minimum absorbance set to zero. After creating a first derivative curve from the raw data, the midpoint temperature of the optical transition (T_m) was determined from the minimum of that curve as described by La Rosa et al [44]. All experiments were performed in 10 mM potassium phosphate buffer, pH 7.5 using 30 μM WT or W45Y amicyanin.

Circular dichroism analysis of the thermal stability of WT and W45Y amicyanins

CD measurements were conducted on WT and W45Y amicyanins in 10 mM potassium phosphate buffer, pH 7.5, for the oxidized and reduced forms, and in 100 mM buffer, for the apo forms because the latter undergoes aggregation at low ionic strength conditions [45]. A 0.4 cm×0.4 cm rectangular quartz cuvette was used and the measurements were done on a J-810 spectropolarimeter equipped with a Peltier temperature controller (Jasco corp., Tokyo, Japan). CD measurements were recorded consecutively on samples which were equilibrated at each temperature for 3 min before measurements. The protein concentration in these experiments was 3 or 7 μM to obtain reasonable signal intensity and still avoid excessive light scattering in the far UV region. The measured ellipticity, θ_{meas} , was converted to mean residue molar ellipticity $[\theta]$, through the formula $[\theta] = \theta_{meas}/nlc$, where $n = 105$ is the number of amino acid residues in the protein, l is the optical pathlength in mm, and c is the molar concentration of the protein. Thermal unfolding of the protein secondary structure was determined from the increase in the negative CD signal at 205 nm which results from an increase in the fraction of unordered structure [46]. Changes in the protein tertiary structure were determined from changes in the signal intensity around 280 nm, generated by the aromatic side chains. In all cases, the value of T_m was determined as the inflection point of

the sigmoidal temperature-dependence curves of the corresponding ellipticity, which was identified as the extremum of the first derivative with respect to the temperature.

Results

Protein expression

Trp45 of amicyanin was converted to alanine, phenylalanine, leucine, lysine, and tyrosine by site-directed mutagenesis. However, only the W45Y amicyanin variant could be isolated from the transformed *E. coli* cells. This suggests that the W45A, W45F, W45L and W45K mutations either disrupted the biosynthesis of amicyanin or decreased its stability such that it could not be expressed or isolated.

Spectroscopic properties

The absorption spectrum of W45Y amicyanin was very similar to that of WT amicyanin. A notable difference is the decrease in absorption at 280 nm and shift of the maximum to 278 nm in the spectrum of W45Y amicyanin (Figure 1A). This is a consequence of the loss of the sole Trp residue in the protein. Each spectrum exhibits an absorption maximum at 595 nm. The extinction coefficient at 595 nm was determined by a quantitative redox titration with a standardized solution of ascorbate and determined to be $4,520 \text{ M}^{-1}\text{cm}^{-1}$ compared to $4,610 \text{ M}^{-1}\text{cm}^{-1}$ for WT amicyanin. Thus, the mutation has negligible effect on the visible absorption. The retention of the strong absorbance at 595 nm is consistent with retention of the type 1 site and strong interaction of Cu(II) with the Cys95 ligand.

Resonance Raman spectroscopy is a sensitive probe of the type 1 copper site and WT amicyanin has been previously characterized by this technique [47]. Excitation of the chromophore produces strong enhancement of fundamental vibrational modes in the 350–450 nm region which have been attributed to Cu-S interaction, as well as overtone and combination bands in the 750–900 nm region [48]. Comparison of the resonance Raman spectra of WT and W45Y amicyanin indicate that they are essentially identical (Figure 1B). This indicates that the mutation has had no apparent effect on the strong Cu(II)-Cys interaction in the type 1 site.

X-ray crystal structures of oxidized and reduced W45Y amicyanin

The X-ray crystal structures of the oxidized and reduced states of W45Y amicyanin were determined to 1.09 Å and 1.02 Å resolution, respectively. Each of these forms was crystallized in the monoclinic space group P21, as has been the case for WT and most other amicyanin variants. Table 1 lists the data collection, refinement and model parameters. The very high-resolution electron density map clearly showed the presence of mutated Tyr45, both in oxidized and reduced forms. The Tyr45 adopts an identical conformation in both forms. The rms deviation between the oxidized forms of native amicyanin (PDB entry 2OV0) and W45Y amicyanin is 0.08 Å with 105 matched C α atoms (Figure 2A) based on the secondary structure matching [49], calculated using CCP4 [39]. The rmsd between oxidized and reduced forms of W45Y amicyanin is 0.15 Å. The copper differs by 0.75 Å between oxidized and reduced forms of W45Y amicyanin. The rmsd between W45Y reduced and WT amicyanin reduced at pH 4.4 and pH 7.7 is 0.32 Å and 0.27 Å respectively (Figure 2B).

There is a presence of negative electron density along with positive density close to His95 ligand around the copper ion site at the reduced state which indicates heightened mobility for the copper ion (~ 2.2 Å from its present position) towards the direction of His95. However, the electron density for each of the ligands of copper ion is well ordered. Attempts to create dual positions for copper ion were not successful (Figure 2C).

The copper coordination distances of WT and W45Y amicyanin at the oxidized and reduced states are shown in Table 2. The positions of Cu(II) and the four ligands are nearly identical in WT and W45Y oxidized amicyanin (Fig. 3A). The B-factors of Cu(II) and Cu(I) are 5.2 Å and 7.0 Å respectively.

On reduction of amicyanin, His95 which serves as a ligand for Cu(II), rotates by 180° about the C β -C γ bond relative to its position in oxidized amicyanin and is no longer in the copper coordination sphere [40]. This accounts for the pH dependence of the E_m value of amicyanin. In the structure of reduced W45Y amicyanin a hydrogen bond between an imidazole nitrogen of His95 and the sulfur of Met51 (3.5 Å) is observed; a feature that is not seen in WT amicyanin (Figure 3B). This may account for the change in the pH-dependence of the E_m value of amicyanin that is caused by the W45Y mutation (discussed later).

In WT amicyanin, the indole ϵ^1 -nitrogen of Trp45 is hydrogen bonded with the hydroxyl η -oxygen of Tyr90 (2.6 Å). This hydrogen bond connects two of the nine β -strands that comprise amicyanin and in doing so may stabilize the overall tertiary structure of the protein. In W45Y amicyanin, this hydrogen bond is lost (Figure 3C). In W45Y amicyanin there is a C-H...O hydrogen bond between methyl group of Val102 and hydroxyl group of Tyr45 (3.4 Å), although this is likely a weaker interaction. These observations may be relevant to the effect of the W45Y mutation on the thermal stability of amicyanin (discussed later).

Redox properties

The E_m value of WT amicyanin was previously shown to be pH-dependent and exhibit a pK_a value of 7.5 ± 0.3 . The E_m value becomes independent of pH at high pH where it exhibits an alkaline pH-independent value of 240 ± 7 mV [40]. The E_m values of W45Y amicyanin was determined by spectrochemical titration over a range of pH values from 6.0–9.0 (Figure 4). The fit of these data to eq 2 yielded a pK_a value of 8.0 ± 0.2 and an alkaline pH-independent value of 236 ± 6 mV, respectively. Thus, it can be concluded that the mutation has caused a small shift in the pH-dependence of the E_m value but has negligible effect on the intrinsic pH-independent E_m value.

Reactivity of W45Y amicyanin with its natural redox partners

The steady-state kinetic parameters for the reactions of W45Y amicyanin with the natural redox partners of WT amicyanin, MADH and cytochrome *c*-551i, were determined and compared with those for the reactions with WT amicyanin. In the reaction of methylamine-dependent reduction of W45Y amicyanin by MADH, values of $k_{cat} = 65.5 \pm 2.8$ s $^{-1}$ and $K_m = 3.2 \pm 0.5$ μ M for W45Y amicyanin were obtained. These were essentially the same as those obtained when the reaction was performed with WT amicyanin which yielded values

of $k_{\text{cat}} = 60.9 \pm 2.1 \text{ s}^{-1}$ and $K_m = 2.3 \pm 0.3 \text{ }\mu\text{M}$. In the reaction of methylamine-dependent reduction of cytochrome *c*-551i by the MADH-W45Y amicyanin complex, values of $k_{\text{cat}} = 12.8 \pm 1.2 \text{ s}^{-1}$ and $K_m = 1.0 \pm 0.2 \text{ }\mu\text{M}$ for W45Y amicyanin were obtained. These values were similar to those obtained when the reaction was performed with WT amicyanin which yielded values of $k_{\text{cat}} = 18.3 \pm 1.7 \text{ s}^{-1}$ and $K_m = 1.3 \pm 0.2 \text{ }\mu\text{M}$. Given the kinetic complexity of this steady-state reaction [43] these data suggest that the W45Y mutation has not significantly affected the reactivity of amicyanin with its natural redox partners.

Effect of the W45Y mutation on the thermal stability of amicyanin

It was previously shown that the temperature dependent bleaching of the visible absorption spectrum of oxidized amicyanin corresponded to the native to unfolded state transition [50]. The loss of absorbance at 595 nm is attributed to the disruption of the type 1 Cu(II) site which gives rise to the strong visible absorption of the oxidized protein. An optical melt experiment was performed with W45Y amicyanin and the results compared with that for WT amicyanin (Figure 5). The W45Y mutation decreased the midpoint temperature at which the loss of visible absorbance occurred from $66.4 \pm 0.2^\circ \text{C}$ in WT amicyanin to $53.9 \pm 0.1^\circ \text{C}$ in W45Y amicyanin.

In order to obtain more detailed information on the structural changes associated with the increase in temperature, temperature-induced changes in the CD spectra of WT and W45Y amicyanins were monitored. The structure of amicyanin consists of β -sheets and short (<10 amino acid residues) loops and turns with no α -helix [8]. As such, the changes in the CD spectrum are relatively simple to interpret and make this an ideal approach to examine the unfolding of the protein. It also allows examination of the reduced and apo forms of the protein which have no absorbance in the visible region. CD has been previously used to monitor the temperature-dependent unfolding of oxidized amicyanin [44].

The CD spectra of both WT and W45Y mutant amicyanin at $T = 37^\circ \text{C}$ display a minimum near 218–220 nm and a maximum of comparable intensity around 195–198 nm, indicative of a β -sheet secondary structure [46] (Figure 6). In addition, a weaker minimum around 280 nm is present that is generated by the aromatic side-chains [51]. A negative CD signal appeared around 200 nm and gained intensity with increasing temperature, in parallel with a reduction of the aromatic side-chain signal at 280 nm, indicating thermal unfolding of the secondary and tertiary structures [46, 51]. The decrease in the β -sheet $n\pi^*$ transition intensity around 218 nm was not always clearly displayed because this effect was largely offset by the substantial increase in the unordered structure band around 200 nm (Figure 6). The thermal transition temperatures (T_m) of the protein secondary structure, as determined from the temperature-dependencies of the CD signal at 205 nm (insets in Figure 6), are summarized in Table 3. This wavelength (205 nm) was chosen to avoid excessive the noise in the CD spectra at lower wavelengths, especially in case of apo proteins which were studied at a higher ionic strength to prevent protein aggregation [45]. Secondary structure unfolding occurred at 60°C for the oxidized WT amicyanin and 53 – 55°C for the reduced and apo forms (Table 3). The W45Y mutation caused significant thermal instability in the oxidized and apo forms of the protein, i.e. a decrease in T_m from 60°C to 46°C and from 55°C to 34°C , respectively, but had less effect on the thermal transition of the reduced protein,

from 53 °C to 50 °C. These data indicate that the W45Y mutation itself exerts strong effect on the thermal stability of the protein's secondary structure.

Tertiary structural transitions are manifested by the decrease in the CD signal at 280 nm, which is generated by the aromatic side chains in the chiral environment of the protein backbone C α atoms [51]. As the tertiary structure opens up with increasing temperature, the chirality of the microenvironment of the aromatic residues and, subsequently, the CD intensity at 280 nm decrease. Temperature-induced changes in the signal at 280 nm paralleled those at 205 nm (Figure 6). Precise interpretation of these effects is complicated by the fact that the unfolded copper-free amicyanin may undergo additional bimolecular reactions. It is known that apoamicyanin has a tendency to form aggregates, particularly at low ionic strength [45] and that the denatured amicyanin may form disulfide dimers between molecules since the lone cysteine residue is free when no longer binding copper [52]. This also affects the thermal reversibility of the unfolding transition [50]. Thus there may be more than one "unfolded" structure and the aromatic residues could have different microenvironments in each structure. Nonetheless, temperature-dependent decrease in the negative ellipticity at 280 nm (Figure 6) indicates thermal unwrapping of the protein's tertiary structure and the analysis showed that these changes precede the secondary structural unfolding.

Discussion

Despite the fact that the copper in the type 1 copper site of amicyanin quenches the fluorescence of Trp45, the replacement of Trp with Tyr has no detectable effect on the electronic or spectroscopic properties of the type 1 copper site. The visible absorbance and resonance Raman spectra, and the pH-independent intrinsic redox potential are unchanged. The structure of the type 1 site with respect to the positions of Cu(II) and the four copper ligands is essentially unchanged. Thus, it appears that Trp45 does not reciprocate any form of electronic communication to the copper. One physical parameter of the type 1 site that is altered by the mutation is the pH dependence of the E_m value. The pH-dependence has been attributed to the fact that His95, which serves as a ligand for Cu(II), when protonated rotates by 180° about the C β -C γ bond and is no longer in the copper coordination sphere of Cu(I) in the reduced protein. The pK $_a$ of W45Y amicyanin is increased by approximated 0.5 pH units. This may be explained by the observation that a hydrogen bond is formed between the rotated and protonated His95 and Met51 in reduced W45Y amicyanin but not in reduced WT amicyanin. This hydrogen bond could stabilize the reduced form in W45Y amicyanin relative to WT amicyanin in a pH-dependent manner thus altering the pK $_a$ value. Flexibility of the ligand loop has been shown to effect the redox and pH-dependent equilibria of cupredoxins [53, 54]. This same structural feature could account for the decreased occupancy of the Cu(I) ion and mobility towards the CD2-His95 ligand to as close as ~1.8 Å, in the crystal structure of reduced W45Y amicyanin (Figure 2C). This structural perturbation may also account for the similar thermal stability of the reduced and oxidized forms of W45Y amicyanin compared to WT amicyanin in which the reduced form is less stable since the hydrogen bond will decrease the mobility of this loop which connects two β -strands.

The most significant effect of the W45Y mutation is on the thermal stability of amicyanin. Decreased thermal stabilities of the oxidized, reduced and apo forms of the W45Y mutant as compared to WT amicyanin were determined from the downshifts in transition temperatures of secondary structure unfolding. The T_m values obtained from the loss of absorbance are approximately 6–7° C higher than those obtained from the secondary structure unfolding determined by CD for both WT and W45Y amicyanin (Table 3). These data suggest that in both the WT and W45Y amicyanin heating first causes tertiary structural distortion when the β -strands move relative to each other, followed by secondary structure disordering, followed by disruption of the type 1 site with loss of copper. From comparison of the crystal structures of WT and W45Y amicyanin it can be inferred that the decrease in overall thermal stability of W45Y amicyanin can most likely be attributed to the loss of a strong interior hydrogen bond between Trp45 and Tyr90 (2.6 Å) in WT amicyanin which links two of the β -sheets.

The importance of the hydrogen bond between Trp45 and Tyr90 is consistent with previous results of a joint x-ray/neutron diffraction study of amicyanin which determined the extent of hydrogen/deuterium exchange and related this to the flexibility and dynamic nature of amicyanin [55]. Trp45 and Tyr90 were among only 14 residues of amicyanin with non-exchangeable hydrogen atoms. Hydrogen bonding to the analogous Trp residue is seen in other cupredoxins. In cucumber stellacyanin (PDB entry 1JER) the indole N of Trp13 is 2.9 Å from the backbone O of Asn47 [20]. In pseudoazurin from *Hyphomicrobium denitrificans* (PDB entry 3EF4) the indole N of Trp50 is within 3.1 Å of the backbone O of Val54 and within 3.2 Å of the backbone O of Ala55 [56]. It was also shown that mutation of Trp48 of *Pseudomonas aeruginosa* azurin decreased the stability of the protein towards guanidine hydrochloride-induced unfolding [57]. The stability afforded to amicyanin by interactions involving Trp45 may be a general feature of β -sheet proteins. For example, the variable domains of immunoglobulins have two β -sheets which are folded face-to-face and have conserved hydrophobic residues clustered along a diagonal pattern of each β -sheet, a feature which has been linked to cupredoxins including amicyanin [58, 59]. The importance of Trp45 in maintaining the structure of amicyanin can further be inferred from the observation that when this residue was mutated to other residues, alanine, phenylalanine, leucine and lysine, no protein could be isolated. This is likely due to an inability to stabilize the properly folded protein.

Acknowledgments

This research was supported by the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health (NIH) under award number R37GM41574 (V.L.D.) and the National Institute of Allergy and Infectious Disease, NIH, under award number 1R03AI097591 (S.A.T). This work and the beamlines 24ID-E and 24ID-C beamlines used to collect data and fluorescence scan are supported by award GM103403 from NIGMS at the NIH. Use of the APS is supported by the U.S. DOE, Office of Science, Office of Basic Energy Science, and Contract No. DE-AC02-06CH1 1357.

ABBREVIATIONS

CD	circular dichroism
E_m	oxidation-reduction midpoint potential

MADH	methyamine dehydrogenase
rms	root mean square
T_m	midpoint temperature for a spectroscopic transition
WT	wild-type

References

- Choi M, Davidson VL. *Metallomics*. 2011; 3:140–151. [PubMed: 21258692]
- Dennison C. *Coord Chem Rev*. 2005; 249:3025–3054.
- Solomon EI, Szilagyi RK, DeBeer George S, Basumallick L. *Chem Rev*. 2004; 104:419–458. [PubMed: 14871131]
- Husain M, Davidson VL. *J Biol Chem*. 1985; 260:14626–14629. [PubMed: 2997215]
- Cunane LM, Chen ZW, Durley RC, Mathews FS. *Acta Crystallogr D Biol Crystallogr*. 1996; 52:676–686. [PubMed: 15299631]
- Husain M, Davidson VL. *J Bacteriol*. 1987; 169:1712–1717. [PubMed: 3558322]
- Husain M, Davidson VL. *J Biol Chem*. 1986; 261:8577–8580. [PubMed: 3013855]
- Durley R, Chen L, Lim LW, Mathews FS, Davidson VL. *Protein Sci*. 1993; 2:739–752. [PubMed: 8495197]
- Chen L, Durley RC, Mathews FS, Davidson VL. *Science*. 1994; 264:86–90. [PubMed: 8140419]
- Chen L, Durley R, Poliks BJ, Hamada K, Chen Z, Mathews FS, Davidson VL, Satow Y, Huizinga E, Vellieux FM, et al. *Biochemistry*. 1992; 31:4959–4964. [PubMed: 1599920]
- Choi M, Sukumar N, Liu A, Davidson VL. *Biochemistry*. 2009; 48:9174–9184. [PubMed: 19715303]
- Davidson VL, Jones LH, Graichen ME, Mathews FS, Hosler JP. *Biochemistry*. 1997; 36:12733–12738. [PubMed: 9335529]
- Davidson VL, Jones LH, Zhu Z. *Biochemistry*. 1998; 37:7371–7377. [PubMed: 9585551]
- Ma JK, Mathews FS, Davidson VL. *Biochemistry*. 2007; 46:8561–8568. [PubMed: 17602663]
- Ma JK, Wang Y, Carrell CJ, Mathews FS, Davidson VL. *Biochemistry*. 2007; 46:11137–11146. [PubMed: 17824674]
- Sun D, Davidson VL. *Biochemistry*. 2003; 42:1772–1776. [PubMed: 12578392]
- Sun D, Li X, Mathews FS, Davidson VL. *Biochemistry*. 2005; 44:7200–7206. [PubMed: 15882058]
- Husain M, Davidson VL, Smith AJ. *Biochemistry*. 1986; 25:2431–2436. [PubMed: 3718960]
- Baker EN. *J Mol Biol*. 1988; 203:1071–1095. [PubMed: 3210236]
- Hart PJ, Nersissian AM, Herrmann RG, Nalbandyan RM, Valentine JS, Eisenberg D. *Protein Sci*. 1996; 5:2175–2183. [PubMed: 8931136]
- Morpurgo L, Finazzi-Agro A, Rotilio G, Mondovi B. *Biochim Biophys Acta*. 1972; 271:292–299. [PubMed: 4340027]
- Szabo AG, Stepanik TM, Wayner DM, Young NM. *Biophysical Journal*. 1983; 41:233–244. [PubMed: 6404322]
- Delfino I, Cannistraro S. *Biophys Chem*. 2009; 139:1–7. [PubMed: 18938024]
- Petrich JW, Longworth JW, Fleming GR. *Biochemistry*. 1987; 26:2711–2722. [PubMed: 3111523]
- Sweeney JA, Harmon PA, Asher SA, Hutnik CM, Szabo AG. *Journal of the American Chemical Society*. 1991; 113:7531–7537.
- Shih C, Museth AK, Abrahamsson M, Blanco-Rodriguez AM, Di Bilio AJ, Sudhamsu J, Crane BR, Ronayne KL, Towrie M, Vlcek A Jr, Richards JH, Winkler JR, Gray HB. *Science*. 2008; 320:1760–1762. [PubMed: 18583608]

27. Engman KC, Sandberg A, Leckner J, Karlsson BG. *Protein Sci.* 2004; 13:2706–2715. [PubMed: 15340166]
28. Davidson VL. *Methods Enzymol.* 1990; 188:241–246. [PubMed: 2126329]
29. Chistoserdov AY, Boyd J, Mathews FS, Lidstrom ME. *Biochem Biophys Res Commun.* 1992; 184:1181–1189. [PubMed: 1590782]
30. Carrell CJ, Ma JK, Antholine WE, Hosler JP, Mathews FS, Davidson VL. *Biochemistry.* 2007; 46:1900–1912. [PubMed: 17295442]
31. Lim LW, Mathews FS, Husain M, Davidson VL. *J Mol Biol.* 1986; 189:257–258. [PubMed: 3783676]
32. Otwinowski Z, Minor W. *Methods Enzymol.* 1997; 276:307–326.
33. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. *J Appl Cryst.* 2007; 40:658–674. [PubMed: 19461840]
34. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC. *Acta Crystallogr D Biol Crystallogr.* 2002; 58:1948–1954. [PubMed: 12393927]
35. Emsley P, Cowtan K. *Acta Crystallogr D Biol Crystallogr.* 2004; 60:2126–2132. [PubMed: 15572765]
36. Brunger AT. *Nature.* 1992; 355:472–475. [PubMed: 18481394]
37. Laskowski R, Thornton J, Moss D, MacArthur M. *J Appl Cryst.* 1993; 26:283–291.
38. Potterton L, McNicholas S, Krissinel E, Gruber J, Cowtan K, Emsley P, Murshudov GN, Cohen S, Perrakis A, Noble M. *Acta Crystallogr D Biol Crystallogr.* 2004; 60:2288–2294. [PubMed: 15572783]
39. CCP4. *Acta Crystallogr Sect D Biol Crystallogr.* 1994; 50:760–763. [PubMed: 15299374]
40. Zhu Z, Cunane LM, Chen Z, Durley RC, Mathews FS, Davidson VL. *Biochemistry.* 1998; 37:17128–17136. [PubMed: 9860825]
41. Cammack, R. *Bioenergetics: A Practical Approach.* Brown, GC.; Cooper, CE., editors. IRL Press; New York: 1995. p. 85–109.
42. Brooks HB, Jones LH, Davidson VL. *Biochemistry.* 1993; 32:2725–2729. [PubMed: 8448129]
43. Davidson VL, Jones LH. *Anal Chim Acta.* 1991; 249:235–240.
44. La Rosa C, Milardi D, Grasso DM, Verbeet MP, Canters GW, Sportelli L, Guzzi R. *Eur Biophys J.* 2002; 30:559–570. [PubMed: 11908848]
45. Davidson VL, Graichen ME, Jones LH. *Biochim Biophys Acta.* 1993; 1144:39–45. [PubMed: 8347660]
46. Sreerama, N.; Woody, RW. *Circular Dichroism: Principles and Applications.* Berova, N.; Nakanishi, K.; Woody, RW., editors. John Wiley & Sons; New York: 2000. p. 601–620.
47. Sharma KD, Loehr TM, Sanders-Loehr J, Husain M, Davidson VL. *J Biol Chem.* 1988; 263:3303–3306. [PubMed: 2830281]
48. Woodruff, WH.; Dyer, RB.; Schoonover, JR. *Biological Applications of Raman Spectroscopy.* Spiro, TG., editor. John Wiley and Sons; New York: 1988. p. 413
49. Krissinel E, Henrick K. *Acta Crystallogr D Biol Crystallogr.* 2004; 60:2256–2268. [PubMed: 15572779]
50. Ma JK, Bishop GR, Davidson VL. *Arch Biochem Biophys.* 2005; 444:27–33. [PubMed: 16289023]
51. Woody, RW.; Dunker, AK. *Circular Dichroism and the Conformational Analysis of Biomolecules.* Fasman, GD., editor. Plenum Press; New York: 1996. p. 109–157.
52. Kumar MA, Davidson VL. *Biotechniques.* 1992; 12:198, 200, 202. [PubMed: 1616709]
53. Battistuzzi G, Borsari M, Canters GW, di Rocco G, de Waal E, Arendsen Y, Leonardi A, Ranieri A, Sola M. *Biochemistry.* 2005; 44:9944–9949. [PubMed: 16026167]
54. Paltrinieri L, Borsari M, Battistuzzi G, Sola M, Dennison C, de Groot BL, Corni S, Bortolotti CA. *Biochemistry.* 2013; 52:7397–7404. [PubMed: 24063705]
55. Sukumar N, Mathews FS, Langan P, Davidson VL. *Proc Natl Acad Sci U S A.* 2010; 107:6817–6822. [PubMed: 20351252]

56. Hira D, Nojiri M, Suzuki S. *Acta Crystallogr D Biol Crystallogr*. 2009; 65:85–92. [PubMed: 19153470]
57. Wilson CJ, Wittung-Stafshede P. *Proc Natl Acad Sci U S A*. 2005; 102:3984–3987. [PubMed: 15753320]
58. Gough J, Chothia C. *Structure*. 2004; 12:917–925. [PubMed: 15274913]
59. Chothia C, Gelfand I, Kister A. *J Mol Biol*. 1998; 278:457–479. [PubMed: 9571064]

Highlights

- Trp45 stabilizes the structure of amicyanin via hydrogen bonding that connects two β -strands
- Trp45 does not influence the electronic properties of copper which quenches its fluorescence
- As temperature is increased disruption of tertiary and secondary structure precedes loss of copper

Figure 1.

A. UV-Visible absorbance spectra of 100 μ M WT (red) and W45Y (blue) amicyanin. B. Resonance Raman spectra of 2 mM WT (red) and W45Y (blue) amicyanin. Each spectrum was recorded in 10 mM potassium phosphate, pH 7.5. The dashed line in A highlights the fact the absorbance maximum of at 280 nm of WT amicyanin shifts in W45Y amicyanin.

Figure 2.

A. Stereoview of the superposition of oxidized W45Y amicyanin (red) with WT amicyanin (PDB code 2OV0; black). The Cu(II) ion is shown as a sphere in blue. Residue 45 of each protein is shown as a ball and stick in respective colors. B. Stereoview of the superposition of reduced W45Y amicyanin (red) with WT reduced amicyanin pH 4.4 (PDB code 1BXA; black) and pH 7.7 (PDB code 2RAC; blue). The Cu(I) ion is shown as a sphere in respective colors. Residue 45 of each protein is shown as ball and stick in respective colors. C. Stereoview of negative density of the Cu(I) site in reduced W45Y amicyanin. The negative

and positive densities at 2.5 σ level are shown in red and black respectively, Cu(I) is shown as a blue sphere and the copper ligands are yellow ball and stick models.

Figure 3.

A. Overlay of the copper sites in oxidized WT amicyanin (green, PDB code 2OV0) and W45Y amicyanin (purple). The copper ion is shown as a blue sphere. B. Overlay of the copper sites in reduced WT amicyanin (green, PDB code 2RAC) and W45Y amicyanin (purple). The copper ion is shown as a blue sphere. The hydrogen bond between His91 and M51 which is observed only in W45Y amicyanin is shown as a dashed line. C. Overlay of the sites surrounding residue 45 in oxidized WT amicyanin (green, PDB code 2OV0) and W45Y amicyanin (purple). The copper ion is shown as a blue sphere. The hydrogen bond between Trp45 and Tyr90 which is observed only in WT amicyanin is shown as a dashed line. Figures were produced using PyMOL (<http://www.pymol.org/>).

Figure 4. Dependence of the E_m value of W45Y amicyanin on pH. E_m values were determined in 10 mM potassium phosphate buffer at the indicated pH at 25 °C. The line is the fit of the data by eq 2.

Figure 5.

The change in absorbance at 595 nm of WT (open circles) and W45Y (closed circles) amicyanin with increasing temperature. For ease of comparison, the absorbance values were normalized so that the maximum absorbance of each is set to 1.0. The data were obtained with 30 μ M protein 10 mM potassium phosphate buffer at pH 7.5. The absorbance data are shown in A and the first derivative of the absorbance versus temperature is shown in B.

Figure 6.

CD spectra of WT (A, B, C) and W45Y (D, E, F) amicyanin as a function of temperature. The protein was in oxidized (A, D), or reduced (B, E), or apo (copper-less) form (C, F). In each case three spectra are shown, those recorded at the lowest temperature (dotted line) the T_m (gray solid line) and highest temperature measured (black solid line). Data for all spectra recorded over the complete range of temperatures are shown in the insets, which present the temperature dependence of the mean residue molar ellipticity at 205 nm (black) and the 1st derivative with respect to temperature (gray).

Table 1

Data Collection and Structure Determination and Refinement

Crystal	W45Y amicyanin (oxidized)	W45Y amicyanin (reduced)
Data collection		
Wavelength (Å)	0.98	0.98
Space group	P2 ₁	P2 ₁
Unit cell dimensions		
a,b,c (Å)	27.2,56.2,28.9	27.2,56.7,28.7
α,β,γ (°)	90,95.9,90	90,96.7,90
Res. limit (Å)	50–1.09 (1.13–1.09)	57–1.02 (1.06–1.02)
I/Sigma(I)	31.8 (15.2)	34.1(11.8)
R _{merge} (%)	5.0 (11.6)	4.4(13.6)
Completeness (%)	93.2 (78.0)	91.7(76.1)
Redundancy	6.8	5.9
Refinement		
resolution range (Å)	25.4–1.09	28–1.02
R-work (%)	12.7	13.4
R _{free} (%)	15.4	15.0
R (working + test) (%)	12.9	13.5
No. of Reflections	33258	40205
Model		
No. of amino acids	105	105
No. of water molecules	174	171
No. of Copper/Sodium	1/1	1/0
Residues in generously allowed region	0	0
Residues in disallowed regions	0	0
No. of residues with alternate conformation	10	2
Stereo-chemical ideality		
bonds (Å)	0.016	0.014
angles (deg)	1.82	1.85
dihedral angles (deg)	10.92	10.63
Planarity (Å)	0.012	0.011

^aValues in parentheses are for the outer shell

Table 2

Copper coordination distances in W45Y and native amicyanin at oxidized and reduced state.

Ligands	W45Y amicyanin oxidized (Å)	a Native amicyanin oxidized (Å)	W45Y amicyanin reduced (Å)	b Native amicyanin reduced pH 4.4 (Å)	c Native amicyanin reduced pH 7.7 (Å)
Cu-SG/Cys92	2.16	2.17	2.11	2.09	2.12
Cu-ND1/His95	2.02	2.05	5.53	5.45	5.33
Cu-ND1/His53	1.97	1.99	1.91	1.91	1.90
Cu-SD/Met98	3.05	3.07	2.99	2.90	2.91

^aPDB entry 2OV0

^bPDB entry 1BXA

^cPDB entry 2RAC

Table 3

T_m values determined from the temperature dependent decrease at 205 nm in the CD spectrum of amicyanin

Amicyanin form	WT amicyanin	W45Y amicyanin
Oxidized	60 ± 1^a °C (66.4 ± 0.2) ^b	46 ± 2 °C (53.9 ± 0.1)
Reduced	53 ± 2 °C	50 ± 1 °C
Apo ^c	55 ± 1 °C	34 ± 2 °C

^a Standard deviations indicate deviations in T_m values in two to three independent experiments.

^b Values in parentheses are T_m values obtained from the optical melt in Figure 4 for comparison.

^c Studies with apoamicyanins were performed in 100 mM buffer to minimize aggregation of the apo forms which occurs in 10 mM buffer, the condition used for the studies of oxidized and reduced amicyanin.