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# Replacement of the axial copper ligand methionine with lysine in amicyanin converts it to a zinc-binding protein that no longer binds copper ☆

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ABSTRACT

The mutation of the axial ligand of the type I copper protein amicyanin from Met to Lys results in a protein that is spectroscopically invisible and redox inactive. M98K amicyanin acts as a competitive inhibitor in the reaction of native amicyanin with methylamine dehydrogenase indicating that the M98K mutation has not affected the affinity for its natural electron donor. The crystal structure of M98K amicyanin reveals that its overall structure is very similar to native amicyanin but that the type I binding site is occupied by zinc. Anomalous difference Fourier maps calculated using the data collected around the absorption edges of copper and zinc confirm the presence of $Zn^{2+}$ at the type I site. The Lys98 NZ donates a hydrogen bond to a well-ordered water molecule at the type I site which enhances the ability of Lys98 to provide a ligand for $Zn^{2+}$. Attempts to reconstitute M98K apoamicyanin with copper resulted in precipitation of the protein. The fact that the M98K mutation generated such a selective zinc-binding protein was surprising as ligation of zinc by Lys is rare and this ligand set is unique for zinc.



## 1. Introduction

Type 1 copper sites are found in a wide range of redox proteins in bacteria, plants and animals, and function as electron transfer (ET) mediators [1]. A single copper is bound in the site and is always coordinated with three strong equatorial ligands; a Cys and two His residues, and with one weak axial ligand, usually a Met. 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 $S(Cys)\pi \rightarrow Cu(II)d_{x2-y2}$ ligand-to-metal charge transfer transition. The physical properties and X-ray crystal structures of several cupredoxins have been characterized [2,3].

Amicyanin is a type 1 copper protein which serves as an electron acceptor for methylamine dehydrogenase (MADH) in many methylotrophic and autotrophic bacteria. Amicyanin from *Paracoccus denitrificans* mediates ET from MADH to cytochrome *c*-551i in the periplasmic space and allows the host bacteria to use methylamine as a sole source of carbon and energy [4]. The complex of MADH, amicyanin, and cytochrome *c*-551i is one of the best characterized physiological protein ET systems. High resolution crystal structures are available for the binary complex of amicyanin with MADH and a ternary complex of MADH, amicyanin and cytochrome *c*-551i [5,6]. Structure-function relationships have been extensively studied by site-directed mutagenesis of amicyanin to identify roles of specific amino acid residues in determining its spectroscopic, redox, and ET properties [3,7]. A high resolution neutron diffraction model for amicyanin has also been reported which revealed a dynamic nature of residues around the copper site and along the ET path [8,9].

The type I copper site of the amicyanin is coordinated by three equatorial ligands provided by nitrogens of His53 and His95 and a sulfur of Cys92, with a fourth axial ligand provided by the sulfur of Met98, thereby forming a distorted tetrahedral geometry [10]. Met98 of *P. denitrificans* amicyanin was previously mutated to Ala, Gln, and Leu [11–14]. These studies indicated that the position and rigidity of the axial ligand of the type 1 copper site influences the overall protein stability, the active site ligation geometry, the spectroscopic properties, and ET properties. The axial ligand was also shown to exert a profound influence on the uptake specificity of the metal ion which occupies the type 1 site.

The role of the metal in the molecular mechanisms of metalloprotein biosynthesis and assembly is not well understood [15]. A comparative study of the specificity of the type 1 site of native and M98Q amicyanins for either $Cu^{2+}$ or $Zn^{2+}$ revealed that the influence of the axial ligand on metal specificity is strongest prior to the completion of protein folding and adoption of the final type I site

☆ Data deposition: The X-ray coordinates of the M98K amicyanin have been deposited in the PDB under code 3RYM.

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geometry [13]. In this study, the role of the axial ligand in determining the metal specificity during and after protein folding was further examined by mutation of Met98 of amicyanin to Lys. This resulted in the isolation of a stable but inactive protein which contained $Zn^{2+}$ in the metal site. In contrast to all other mutants of amicyanin which have been characterized, it was not possible to remove the zinc and then reconstitute M98K amicyanin with copper. The structure of this unusual zinc-specific amicyanin is presented together with data concerning its ability to interact with MADH, and properties of the metal-free M98K apoamicyanin.

## 2. Materials and methods

### 2.1. Protein purification

Previously described procedures were used to purify native amicyanin [4] and MADH [4] from *P. denitrificans*. M98K amicyanin was expressed in *Escherichia coli* and purified from the periplasmic fraction as described previously for recombinant wild-type amicyanin [16].

### 2.2. Site-directed mutagenesis of the amicyanin gene

Site-directed mutagenesis was performed on double-stranded pMEG201[16] using two mutagenic primers with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotide sequences used to construct M98K were 5'-GACTATCACTGTACCCCG-CATCCCTTCAAGCGCGGCAAGGTCG-3' and its complementary DNA. The underlined bases are those that were changed to create the desired mutation. The entire 555-base *mauC*-containing fragment was sequenced to ensure that no second site mutations were present, and none were found.

### 2.3. Preparation of completely unfolded, partially folded, and fully folded apoamicyanin

M98K amicyanin was subjected to a procedure which was previously developed to remove $Zn^{2+}$ from other amicyanin mutants and then to reconstitute those proteins with $Cu^{2+}$ [14,17]. To completely unfold the protein and remove any metal ions, M98K amicyanin was incubated in 10 mM HEPES buffer, pH 8.0, containing 6 M guandinine–HCl, 50 mM EDTA and 2 mM dithiothreitol. The completely unfolded apoprotein was then diluted 50-fold into 10 mM HEPES buffer, pH 8.0, containing 5 mM dithiothreitol. The protein was then dialyzed against 100 mM ammonium acetate, pH 8.0, at 4 °C for 4 h, followed by dialysis overnight against 250 mM ammonium acetate, pH 8.0, to generate the partially folded protein. The fully folded apoprotein was prepared by dialysis of the holoprotein for 20 h at 4 °C against 0.1 M Tris–HCl, pH 8.0, containing 0.1 M KCN, followed by dialysis against 0.05 M potassium phosphate, pH 7.5, to remove KCN and unbound metal, as previously described [18]. This native apo-amicyanin was shown by X-ray crystallography to have a structure essentially identical to that of holoamicyanin except for the absence of copper [10]. Reconstitution experiments with the unfolded, partially folded and fully folded M98K amicyanin apoproteins were performed at room temperature by incubation with $CuSO_4$.

### 2.4. Test for reactivity of the single Cys sulfhydral group of amicyanin

The reactivity of the free sulfhydral of the sole Cys of amicyanin that provides one of copper ligands was probed with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The reaction with DTNB was quantitated from the increase in absorbance at $A_{412}$ nm with an extinction coefficient of 13600 $M^{-1}$ $cm^{-1}$ as described previously [18].

### 2.5. Steady-state kinetic assays

Steady-state kinetic studies of the MADH-catalyzed reduction of native amicyanin by methylamine were performed as described previously [19]. Assay mixtures contained 18 nM MADH and varying concentrations of amicyanin in 10 mM potassium phosphate buffer at pH 7.5 at 30 °C. The reaction was initiated by the addition of 0.1 mM methylamine. As M98K amicyanin showed no activity in this reaction, it was tested as an inhibitor of the reaction with native amicyanin. In those studies the reactions with native amicyanin were repeated in the presence of different fixed concentrations of M98K amicyanin. Activity was monitored by the change in $A_{595}$ nm caused by the reduction of amicyanin. Initial rates were measured from the linear portion of the progress curve and fit according to the standard Michaelis-Menten equation to extract $k_{cat}$ and $K_m$ values. The data for the inhibition of the reaction by M98K were best fit to Eq. (1), where $K_i$ is the simple dissociation constant for the M98K amicyanin–MADH complex.

$$1/v = (K_m/V_{max})(1 + [\text{M98K amicyanin}]/K_i)(1/[\text{amicyanin}]) + 1/V_{max} \quad (1)$$

### 2.6. Crystallization

Prior to crystallographic trials, the M98K mutant of amicyanin was dialyzed against 5 mM sodium monobasic/potassium dibasic phosphate buffer, pH 6.6. As this mutant failed to crystallize using previously used protocols which were successful with native amicyanin and other mutant amicyanins [20,21], a wide range of crystallization conditions was screened. The M98K amicyanin crystals were grown by the sitting drop vapor diffusion method, by mixing equal volumes of protein (13.8 mg/ml) and reservoir solution. The reservoir solution contained 0.01 M zinc sulphate heptahydrate, 0.1 M MES pH 6.5 and 25% w/v PEG monomethylether 550. Crystals suitable for X-ray diffraction analysis were cryoprotected with frombin oil (purchased from Sigma).

### 2.7. Data collection

X-ray diffraction data for M98K amicyanin was collected to 1.7 Å resolution at the NE-CAT beamline 24ID-C at the Advanced Photon Source, Argonne National Laboratory, Argonne equipped with Micro-diffractometer-MD2 and ADSC Quantum 315 CCD detector. A fluorescence scan was carried out at the Zn and Cu absorption energy to verify the presence of Zn or Cu ion and the Zn scan showed clear peak indicating the presence of Zinc. In order to verify the presence of Cu or Zn ion at the type I site and to differentiate the contribution of zinc and copper ions from other elements including sulfur which is present naturally in the protein, data were collected around the copper and zinc absorption edges. The data collection energy for Zn was chosen from Zinc scan while copper foil fluorescence scan was used to choose data collection energy for Cu, as the Cu scan does not show any clear peak. For copper, data were collected at 1.3869 Å (below-peak), 1.3776 Å (peak) and 1.3700 Å (above-peak) and for zinc at 1.2861 Å (below-peak), 1.2819 Å (peak) and 1.2749 Å (above-peak). The data were processed, scaled and merged in HKL2000 [22]. The M98K amicyanin crystallized in the triclinic space group (P1) which is different than that was observed for previously crystallized native and mutant amicyanins. The unit-cell parameters and diffraction statistics are listed in Table 1.

### 2.8. Structure solution and refinement of M98K amicyanin

The M98K amicyanin structure was solved by molecular replacement method using PHASER [23] of PHENIX [24] using the coordinates for native amicyanin (PDB id 1AAC) with Met98 mutated to Ala. There were four molecules in the asymmetric unit. Inspection of the resulting