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Non-covalent interactions in blue copper protein probed by Met16 mutation and electronic and resonance Raman spectroscopy of *Achromobacter cycloclastes* pseudoazurin

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ABSTRACT

We have used low-temperature (77 K) resonance Raman (RR) spectroscopy as a probe of the electronic and molecular structure to investigate weak π–π interactions between the metal ion-coordinated His imidazoles and aromatic side chains in the second coordination sphere of blue copper proteins. For this purpose, the RR spectra of Met16 mutants of *Achromobacter cycloclastes* pseudoazurin (*Ac*PAz) with aromatic (Met16Tyr, Met16Trp, and Met16Phe) and aliphatic (Met16Ala, Met16Val, Met16Leu, and Met16Ile) amino acid side chains have been obtained and analyzed over the 100–500 cm^{-1} spectral region. Subtle strengthening of the Cu(II)–S(Cys) interaction on replacing Met16 with Tyr, Trp, and Phe is indicated by the upshifted (0.3–0.8 cm^{-1}) RR bands involving $\nu(Cu–S)_{Cys}$ stretching modes. In contrast, the RR spectra of Met16 mutants with aliphatic amino acids revealed larger (0.2–1.8 cm^{-1}) shifts of the $\nu(Cu–S)_{Cys}$ stretching modes to a lower frequency region, which indicate a weakening of the Cu(II)–S(Cys) bond. Comparisons of the predominantly $\nu(Cu–S)_{Cys}$ stretching RR peaks of the Met16X = Tyr, Trp, and Phe variants, with the molar absorptivity ratio $\varepsilon_1/\varepsilon_2$ of $\sigma(\sim455\ nm)/\pi(\sim595\ nm)$ (Cys)S → Cu(II) charge-transfer bands in the optical spectrum and the axial/rhombic EPR signals, revealed a slightly more trigonal disposition of ligands about the copper(II) ion. In contrast, the RR spectra of Met16Z = Ala, Val, Leu, and Ile variants with aliphatic amino acid side chains show a more tetrahedral perturbation of the copper active site, as judged by the lower frequencies of the $\nu(Cu–S)_{Cys}$ stretching modes, much larger values of the $\varepsilon_1/\varepsilon_2$ ratio, and the increased rhombicity of the EPR spectra.

1. Introduction

Pseudoazurin (PAz) belongs to a family of blue (type 1) copper proteins that act as electron-transfer (ET) agents in the respiratory chains of denitrifying bacteria and various plants [1,2]. In numerous microorganisms, PAz serves as an electron donor to nitrite reductase (NiR) that carries out the reduction of NO_2^- to NO under anaerobic conditions [3]. The oxidized copper center of PAz displays a number of unusual properties, including an intense absorption centered around 594 nm ($\varepsilon = 3700\ M^{-1}\ cm^{-1}$) arising from (Cys)S → Cu(II) charge transfer (CT), relatively high redox potential (260 mV) and a distinctive rhombic EPR spectrum with a small hyperfine coupling constant (~55 G) [4]. Up to this date, several X-ray crystal structures of pseudoazurins from various sources, including *Achromobacter cycloclastes* (*Ac*) [5], *Alcaligenes faecalis* (*Af*) [6,7], *Methylobacterium extorquens* (*Me*) [8], *Thiosphaera pantotropha* (*Tp*) [9], and *Hyphomicrobium denitrificans* (*Hd*) [10], have been determined.

The crystal structure of *Ac*PAz revealed that the protein consists of a single polypeptide chain folded in an eight-stranded β-barrel structure (Fig. 1a), which resembles the overall topology of the other type 1 copper (T1Cu) sites [11–13]. In contrast to other blue copper proteins, all pseudoazurins also possess two C-terminus α-helices. The metal binding site consists of a single copper atom, that is located approximately 5 Å beneath the protein surface and shows a distorted tetrahedral geometry. The copper atom is coordinated by the N^{δ} atoms of two histidines (His40, His81) and

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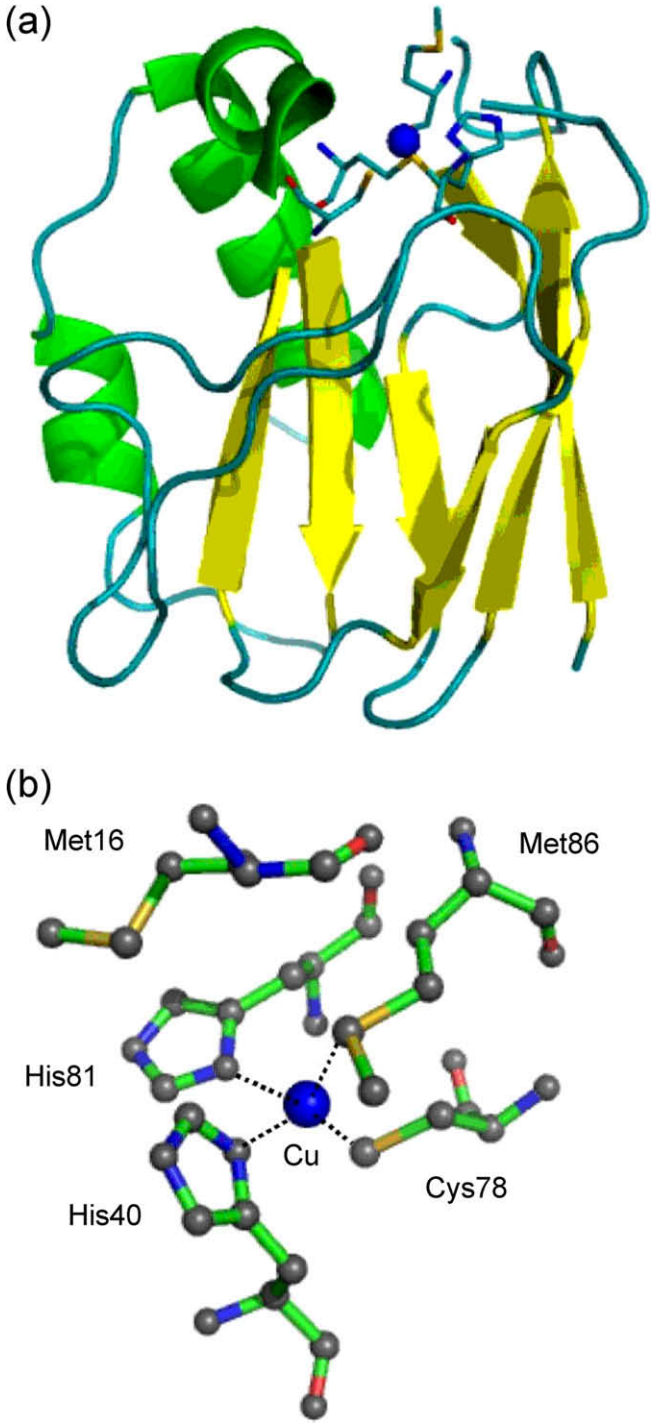

Fig. 1. Protein (a) and copper site (b) structures of *A. cycloclastes* pseudoazurin (PDB: 1bqk). These pictures were drawn with the program PyMol.

a thiolate sulfur of cysteine (Cys78), and shows a displacement of ~0.4 Å from the N_2S plane toward the weaker axial methionine (Met86) residue (Fig. 1b) [5]. In addition, the ligated to Cu(II) His81 interacts weakly with the side chain of Met16 residue, which is in the close vicinity of the PAz–NiR binding site [14–16].

Two independent concepts introduced by Williams (entatic state) [17] and Malmström (rack-induced bonding) [18], proposed that the second coordination sphere of the rigid protein matrix influences the properties of the copper active site. The most interesting feature of the second coordination sphere effect has been focused on the H-bonding in protein molecules [19–23]. Recently, weak interactions of the side chain aromatic rings (cation–π and π–π interactions) became a subject of interest. These interactions were found to play an important role in stabilizing the tertiary structure of proteins, and possibly influencing their metal active sites [24–27]. The importance of aromatic ring stacking interactions involving proteins and nucleic acids has been extensively studied over the past decade in biological systems, including α-amino acids [26,28], horseradish peroxidase [29], *Pseudomonas putida* dioxygenase [30], plastocyanins from fern *Dryopteris crassirhizoma* [31–33], spinach [34] and *Phormidium laminosum* [34], galactose oxidase [35], metalloporphyrins [27], and various model complexes [24,25].

As mentioned earlier, in the crystal structure of *Ac*PAz, the solvent-exposed His81 interacts weakly with the side chain of Met16, which was found to be located in the close vicinity of the alanine 15 residue (Ala15) [5]. Recent NMR spectroscopic studies revealed that Ala15 could be involved in the formation of the *Ac*PAz–NiR complex [14,15]. Therefore, an investigation of the His81–Met16 interaction became necessary [36] in order to understand its role in modulation of the electronic structure and reactivity of the blue copper(II) site.

Previously, some of us have prepared a series of Met16X (*X* = Tyr, Trp, and Phe) *Ac*PAz variants with aromatic acids in order to explore potential π–π interactions involving metal ion-coordinated ligands [34,36–39]. The Met16Val pseudoazurin with an aliphatic amino acid chain has also been constructed to eliminate the effect of such interactions [38,39]. The π–π stacking interactions between the solvent-exposed His81 imidazole and the side chain group of aromatic and aliphatic amino acid residues have been investigated by electronic absorption, circular dichromism (CD), electron paramagnetic resonance (EPR), ^{1}H nuclear magnetic resonance (NMR), and ultraviolet resonance Raman (UVRR) spectroscopic methods, as well as cyclic voltammetry [37–39]. These combined experimental results revealed that the π–π stacking interaction between the coordinated histidine imidazole and the aromatic amino acids is an important mechanism in modulating the structure and reactivity of the blue copper site. Very recent X-ray crystallographic studies of the Met16Phe *Ac*PAz protein also demonstrated the remarkable effect of the π–π interaction at the active site [34]. Moreover, the Met16Val mutant was found to be less stable than the wild type (WT) *Ac*PAz and its aromatic acid introduced mutants, and the importance of the weak interactions at Met16 position was reported [39].

The prime focus of the present work is to explore structural properties and bonding interactions at the *Ac*PAz T1Cu site by using low-temperature (77 K) resonance Raman (RR) spectroscopy with visible excitation [40]. RR spectroscopy has been a very powerful technique in selectively probing CT chromophores of blue copper proteins [41–46], hence providing a more detailed description of copper(II)–ligand structures.

Herein, we report the detailed results of electronic absorption, RR scattering, and electrochemistry from a series of *A. cycloclastes* pseudoazurins in which Met16 has been replaced by aromatic and aliphatic amino acids: tyrosine (Met16Tyr), tryptophane (Met16Trp), phenylalanine (Met16Phe), valine (Met16Val), leucine (Met16Leu), alanine (Met16Ala), and isolucine (Met16Ile). The experimental data show the advantage of using the RR spectroscopy to obtain structural information regarding the nature of subtle variations in copper(II)–amino acid interactions caused by Met16 replacements. It was possible to evaluate these interactions by structural interpretation of the Cu(II)–N(His) and Cu(II)–S(Cys) stretching frequencies of *Ac*PAz mutants. The changes observed in the RR spectra of Met16 variants showed a subtle strengthening (shortening) of the Cu(II)–S(Cys) bond in Met16X = Tyr, Trp, and Phe variants relative to the wild type (WT) and Met16Z = Ala,