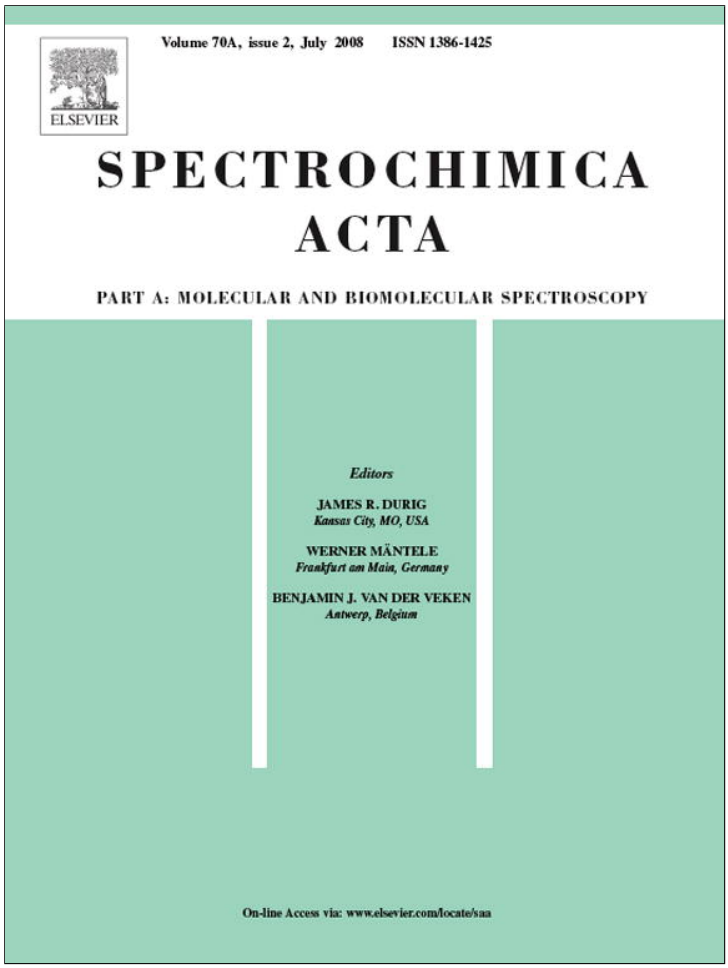

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The effect of Trp83 mutant on the properties of CopC

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Abstract

CopC, a protein involved in copper resistance, is essentially constituted by two sheets forming a Greek key β barrel motif. The aromatic ring of Trp83, sandwiched between the two β sheets, has numerous contacts with residues in strands β and stabilizes the protein fold. In the paper Trp83 was mutated to Leu to study the effect of this mutation on CopC by means of fluorescence spectra and UV spectra. The experiments indicate that the mutation bind Cu^{2+} with a decreased formation constant of $3.95 \times 10^{11}\ M^{-1}$ in 20 mM PB buffer at pH 7.0; mutagenesis make hydrophobic region to be exposed to an extent. Compared with the wild, thermal stability of the mutant was shown to decrease by stronger fluorescence of TNS at 80 °C. The important role of aromatic residue in structure is exhibited.

Keywords: CopC; Site-mutant; TNS

1. Introduction

Copper is an essential trace element required for survival by all organisms from bacterial cells to humans. It serves as important catalytic cofactors for many proteins and enzymes that carry out fundamental biological functions that are necessary for growth and development [1]. However, Cu ion is also very toxic in the free form because of its ability to produce radical by cycling between oxidized Cu(I) and reduced Cu(II) [2]. Therefore, intracellular copper ion levels in living organisms are tightly controlled by subtle molecular mechanism to attain homeostatic balance [3–5].

Among these mechanisms, sequestration of copper by periplasmic and outer membrane proteins as a resistance mechanism in the plant pathogen *Pseudomonas syringae* (apparently different from copper resistance mechanism of enhancing copper efflux in *E. coli* [6]) has been proposed [7]. Originally isolated from strains of *P. syringae* that infects tomato in California [8,9], the plasmid-borne copper resistance operon consists four genes (CopABCD) [10], under the control of a copper-inducible promoter. Analysis of Cop proteins suggests the model for copper resistance in which free copper ions are prevented from entering the cytoplasm (CopD plays the role) by compartmentalization in the periplasm (CopA, CopC for it) and out membrane (CopB for it) [11,12].

CopC, accounting for almost 1% of total cellular protein in induced cell, is a soluble protein of 102 amino acids with molecular weights near 10,000. In solution it adopts a fold essentially constituted by nine β strands forming a barrel motif. Known copper(II) site located in N-terminal end of the barrel is constituted by His-1, Glu-27, Asp-89 and His-91, about 30 Å apart from copper(I) site located in C-terminal [13]. Two distinct copper binding sites are highly specific for both Cu(I) and Cu(II), which maintains free copper ion concentrations in solution at sub-picomolar levels. The aromatic ring of Trp83, sandwiched between the two β sheets, has numerous contacts with residues in strand β2 and strand β7 and stabilizes the protein fold [13]. CopC had been reported as a redox switch [14] as well as copper trafficking mechanism was published [15,16]. In the paper some properties of Trp mutant of CopC were investigated to understand the role of aromatic residues in stabilizing the structure of CopC.

2. Material and methods

2.1. Material

Potassium dihydrogen phosphate, potassium hydrogen phosphate and phosphoric acid, metallic compound were all

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analytical reagents. Tryptone, yeast extract, ampicillin, chloramphenicol, isopropyl-β-D-thiogalactopyranoside (IPTG) and sodium chloride were got from Sangon Ltd. CM-Sephaddex (C-25) was got from Pharmacia. Cellulose (DE-52) was got from Whatman.

The instruments are Hewlett Packard 8453 spectrophotometer, F-2500 fluorescence spectrophotometer, Hitachi 850 fluorescence spectrophotometer and pH meter.

2.2. Methods

2.2.1. Plasmids and site-mutant

The pTE-20b-CopC plasmid was a kind gift from Dr. A.G. Wedd, University of Melbourne. Mutant of CopC gene was carried out by PCR targeting the intended mutation site with overlapping primers carrying the designed variation. The variant generated was confirmed by DNA sequencing.

2.2.2. Protein expression and purification

ApoCopC protein was expressed and isolated essentially as reported [13] with an extra gelfiltration purification step. Negatively charged *E. coli* proteins were removed by passing the lysate supernatant through a DE-52 anion exchange column in 20 mM PB buffer at pH 8. The flow-through fraction was adjusted to pH 6 with 0.2 M PB and then applied to a CM cation exchange column in 20 mM PB at pH 6. The bound proteins were eluted with a salt gradient of 0–0.2 M NaCl in 20 mM PB buffer. The final purified protein was detected by 15% SDS-PAGE and less than 1% Cu content was obtained by atomic absorption spectrum.

2.2.3. Protein concentration

The protein concentration was measured spectrophotometrically using molar extinction coefficients at 280 nm of 6970 M^{-1} cm^{-1} for wild CopC and 278 nm of 1400 M^{-1} cm^{-1} for the mutant CopC (MCopC). The extinction coefficient of CopC was estimated from the tyrosine (Tyr) and tryptophan (Trp) content as described by Pace et al. [17].

2.2.4. Thermal stability measurements

Thermal denaturation experiments were performed in 20 mM phosphate buffer, pH 7.0, at a protein concentration of 5.5 μM. The temperature of the solutions was maintained by a jacketed cell holder connected to an external circulating water bath (Shimadzu TB-85) under air atmosphere with a heating rate of 0.3 °C min^{-1}, ranged from 25 to 100 °C. The spectra measurements were performed in 1 mL quartz cuvettes.

3. Results and discussion

3.1. Binding cupric

Copper(II) site in wild CopC located in N-terminal of the barrel is constituted by His-1, Glu-27, Asp-89 and His-91, which was expected not to be affected on by tryptophan mutant located in hydrophobic core. At first, UV difference spectroscopy of copper titrating mutant CopC was monitored. MCopC contains

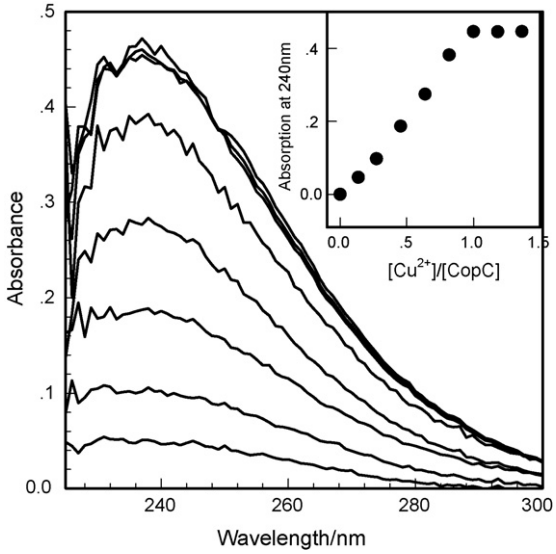

Fig. 1. UV difference spectra of Cu^{2+} (1 mM) titration MCopC (55.0 μM) in 2 mL PB (20 mM) buffer, pH 7.0. The volume of cupric is 15, 30, 50, 70, 90, 110, 130, 150 μL, respectively. *Inset*: Titration curve of Cu^{2+} to MCopC.

no tryptophan and has only tyrosine at position 79 and three phenylalanine at 25, 33 and 98. Cu^{2+}-induced alterations to absorption of chromophores in UV spectroscopy displayed interactions between protein and the metal. Fig. 1 showed that the change of an apparent Cu^{2+}-induced in absorbance at 240 nm gradually increased untill the ratio of 1:1 achieved. Apparently the mutant still binds one equiv. of copper.

In order to test whether binding constant is influenced on or not, MCopC was added in the mixture of wild CopC and copper with 1:1. Fig. 2 displayed fluorescence change of wild CopC with the addition of different concentration of MCopC. As the concentration of MCopC was increased, fluorescence of CopC at 320 nm quenched by Cu^{2+} gradually restored. To avoid tyrosine absorption, the excitation wavelength of 295 nm was chosen. So

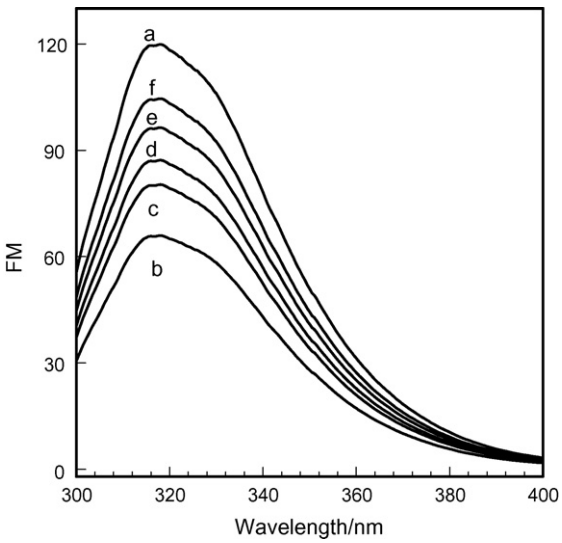

Fig. 2. CopC fluorescence change spectra in 1 mL PB (20 mM) buffer, pH 7.0. (a) ApoCopC (2.75 μM); (b) adding 27 μL Cu^{2+} (0.1 mM). (c–f) Adding MCopC (67 μM) 100, 150, 250, 350 μL.

fluorescence increase absolutely arose from copper transferring from the wild to the mutant. The reaction occurred:

$$\mathrm{CopC-Cu} + \mathrm{MCopC} \leftrightarrow \mathrm{MCopC-Cu} + \mathrm{CopC}$$

So the ratio of restored fluorescence intensity to whole fluorescence quenched by one equiv. of Cu^{2+} was proportional to the concentration percentage of free CopC in total CopC:

$$\frac{[\mathrm{CopC}]_f}{[\mathrm{CopC}]_t} = \frac{F - F_{Cu^{2+}}}{F_0 - F_{Cu^{2+}}}$$

According to it and mass balance equations for cupric, CopC, MCopC:

$$[\mathrm{CopC-Cu}]_f = [\mathrm{CopC-Cu}]_t - [\mathrm{CopC}]_f$$

$$[\mathrm{MCopC}]_f = [\mathrm{MCopC}]_t - [\mathrm{MCopC-Cu}]_f$$

The binding constant of MCopC to cupric could be calculated:

$$K_{\mathrm{MCopC-Cu}} = \frac{[\mathrm{MCopC-Cu}]_f[\mathrm{CopC}]_f}{[\mathrm{CopC-Cu}]_f[\mathrm{MCopC}]_f} \times K_{\mathrm{CopC-Cu}}$$

To correct for dilution during each titration and normalize the results from different titration, the fluorescence intensity was converted to molar fluorescence intensity (F_M) by dividing the fluorescence intensity vs. the analytical concentration of CopC. The binding constant K of $3.95 \times 10^{11}\ M^{-1}$ could be obtained. (The wild binding copper constant $K_{CopC-Cu}$ of 1.8×10^{13} was chosen [18].)

NMR and crystal structure analysis indicated: in β-barrel structure of CopC, the aromatic ring of Trp83, belonging to strand β6, is sandwiched between two sheets as it has numerous contacts with residues in strands β2 (sheet 1) and β7 (sheet 2). The metal ion location is within a sphere of about 10 Å centered on a position equidistant from the loops connecting strands β2 and β3 and strands β6 and β7. Did the substitution of tryptophan with leucine involve in the movement of both strands and side-chain atoms, subsequently give rise to change from ligand residues to some extent? Most similar single mutant maintained a native-like fold [19,20]. MCopC should be coincident to them. Maybe slight strands movement led to geometry distortion of the active site of the protein and consequently protein deviated from intrinsic binding capacity.

3.2. Effect on the conformation

2-*p*-Toluidinynaphthalene-6-sulfonate (TNS) is a hydrophobic probe that hardly fluoresces in water but dramatically enhances fluorescence intensity and blue shifts in organic solvents and when bound to certain protein [21]. It has been utilized at all times to inspect the hydrophobic domain of protein. The soluble CopC constituted by nine β strands, forming a Greek key β barrel motif. The accessibility of hydrophobic core of barrel in the CopC was judged by adding TNS. Fig. 3 showed TNS fluorescence sensitivities in same concentration of the wild and the mutant, respectively. It could be seen that the emission maximum of TNS in the protein was blue-shifted by comparing with emission maximum at 500 nm in aqueous solution and

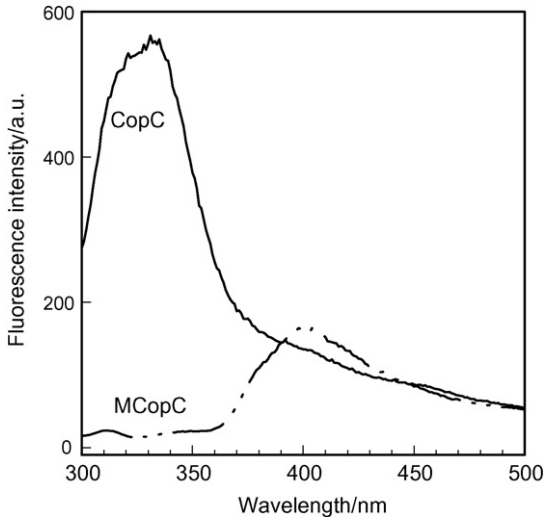

Fig. 3. TNS (35 μM) emission in CopC (5.5 μM) and MCopC (5.5 μM), respectively.

located at 400 nm. For the wild, TNS emission was inconspicuous while it was obvious for the mutant, which was a signal that the hydrophobic domain of MCopC exhibited easier accessibility for the probe while the hydrophobic domain of the wild could hardly be attacked. This was proved further in Fig. 4 when TNS was added in the two proteins with one equiv. of copper. Binding copper made the protein more sensitive to TNS both for the wild and for the mutant, which was shown by the further increase of TNS fluorescence at 400 nm. Metal load made hydrophobic region of protein "open" to an extent so that TNS accessed to it more easily than to apo-type just as phenomena was found in many proteins [22,23]. But difference still existed for CopC and MCopC: TNS exhibited stronger sensibility in mutant MCopC – Cu solution than wild CopC – Cu. The metal-dependent changes of the degree of hydrophobic accessibility to CopC and MCopC were obtained. Both accordant experiments proved that the introduction of leucine in place of an aromatic

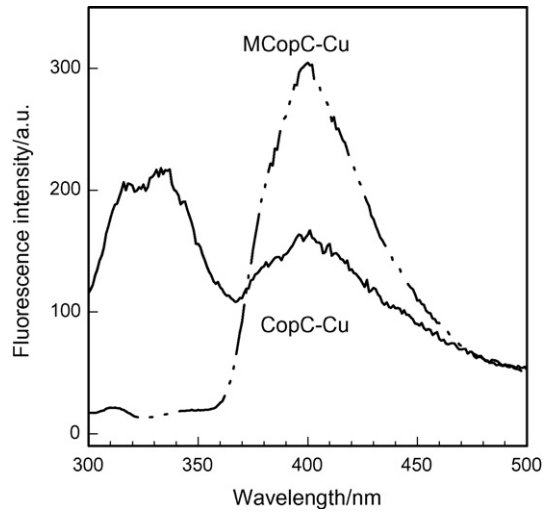

Fig. 4. TNS (35 μM) emission in CopC – Cu (5.5 μM) and MCopC – Cu (5.5 μM), respectively.

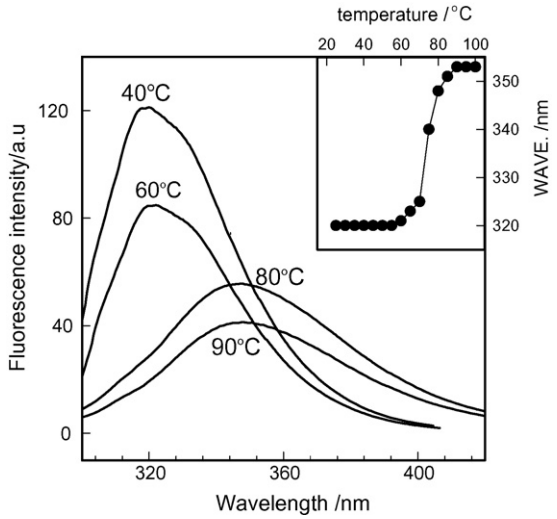

Fig. 5. Tryptophan emission of apoCopC (5.5 μM) in different temperature. Insert plot represents denaturation curve of CopC, wavelength at maximum emission vs. temperature.

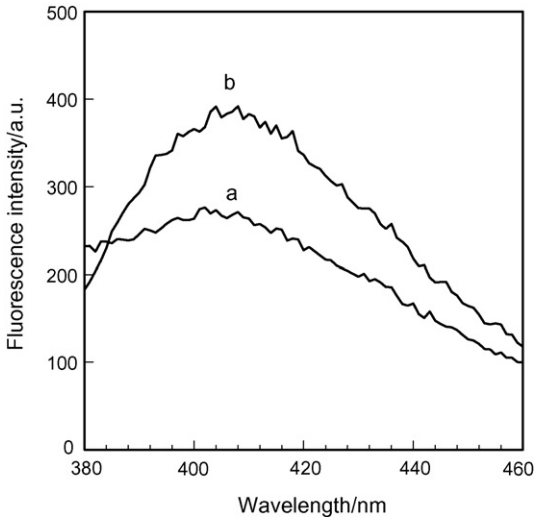

Fig. 6. Emission of TNS (35 μM) in the concentration of 5.5 μM CopC (curve a) and MCopC (curve b) at 80 °C, respectively.

tryptophan had created accessibility to hydrophobic region so that TNS more easily accessed to hydrophobic region of the mutant whether with metal or not. Ser in place of a bulky Phe in azurin created a cavity of ~40 Å^3 in I7S mutant [15]. Maybe forming similar cavity helped TNS attack to the hydrophobic region.

3.3. Thermal stability

Although thermal denaturation is not strong enough to break the peptide bonds, it involves the disruption and possible destruction of both the secondary and tertiary structures. In a sense, thermal denaturation makes protein unfold to an extent and exposes side chains of hydrophobic residues embedded inside native structure. So structure change of protein can be obtained by monitoring the exposure of hydrophobic residues. CopC change with increasing temperature was shown in Fig. 5 by emission change of tryptophan. Obviously, exposure of hydrophobic residues increased with the protein unfolding going which could be shown by gradually red-shifted emission of tryptophan. The Gibbs free energy of CopC was rough calculated according to two-state model by13.0 kJ M^{-1}.

For the mutant, there is no tryptophan and TNS was chosen to probe exposure of hydrophobic residues to obtain information about structure change. Fig. 6 showed TNS fluorescence in both wild CopC and mutant MCopC at 80 °C to achieve qualitative compare. With temperature rose, both proteins exhibited increased exposure of hydrophobic residues by enhancing fluorescence intensity of TNS, but fluorescence intensity of TNS in MCopC at 80 °C was 2.5 times as much as at room temperature while it was 2.1 times in CopC. Compared with the wild, TNS in MCopC solution has stronger fluorescence increase. The exposure of hydrophobic residues correlated to the loss of tertiary structure, so it can be concluded that MCopC thermal stability is inferior to CopC.

4. Conclusion

Compared with wild CopC, tryptophan site mutagenesis exhibited properties change in some aspects. Binding constant of MCopC to Cu^{2+} decreases and was caulated to 3.95×10^{11} M^{-1} in 20 mM PB buffer at pH 7.0 and the accessibility to the hydrophobic domain turned easier by TNS increasing sensibility. Under same denatured temperature of 80 °C, difference of TNS fluorescence showed that the loss of structure was larger in the mutant than in the wild. In general, key role of aromatic residue Trp in CopC was shown in this paper.

Acknowledgements

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