Journal of Inorganic Biochemistry 159 (2016) 45–49

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Copper(I) stabilization by cysteine/tryptophan motif in the extracellular domain of Ctr4

Mariko Okada, Takashi Miura *

Graduate School of Pharmaceutical Sciences, Tohoku University, Aobayama, Sendai 980-8578, Japan

ARTICLE INFO

Article history:
Received 18 September 2015
Received in revised form 15 January 2016
Accepted 10 February 2016
Available online 11 February 2016

Keywords:
Ctr4 copper transporter
Metal coordination
Cysteine
Tryptophan
Cation–π interaction
Raman spectroscopy

ABSTRACT

Copper transporter Ctr4 of fission yeast has a quasi-palindromic sequence rich in cysteine and aromatic amino acid residues, $CX_4YWNWYX_4C$ (where X represents any amino acid), in the N-terminal extracellular domain. A 24-mer peptide comprising this sequence is bound to Cu(I) through the cysteine thiolate coordination. Luminescence, UV absorption and resonance Raman spectra of the Cu(I)-peptide complex show that at least one of the two tryptophan side chains is located in close proximity to the thiolate-Cu(I) center and interacts with the Cu(I) ion via π-electrons of the indole ring. Although the thiolates and Cu(I) are oxidized to disulfide and Cu(II), respectively, only very slowly in air-saturated solutions, replacements of the tryptophan residues to phenylalanine significantly accelerate the oxidation reactions. The results obtained indicate that the interaction between Cu(I) and tryptophan via π-electrons plays a significant role in protecting the thiolate-Cu(I) center against the oxidation. The cysteine- and tryptophan-rich quasi-palindromic sequence may be a metal binding motif that stabilizes Cu(I) in the oxidizing extracellular environment.

1. Introduction

Copper is a trace element essential for normal cell homeostasis and can adopt both Cu(I) and Cu(II) oxidation states in living organisms. Eukaryotic cells acquire copper from the extracellular space via integral membrane proteins of the Ctr (copper transporter) family [1,2]. Since the copper in the reduced Cu(I) state is employed in the intracellular copper trafficking [3], the N-terminal domains of Ctr proteins are required to capture Cu(I) in the substantially oxidizing extracellular environment and to recruit it to the transmembrane pathway [4]. The methionine-rich motifs (Mets motifs), MXXM and MXM, which are conserved in the N-terminal domains of Ctr proteins, have recently been recognized as major binding sites for Cu(I) in the oxidizing extracellular environment [5,6]. Thiolate of cysteine rarely acts as ligand for Cu(I) in the extracellular space mainly due to its propensity to form disulfide bond [6]. For example, no cysteine residue is found in the methionine-rich extracellular domain of Ctr1 proteins of a variety of species from budding yeast *Saccharomyces cerevisiae* to human, though there are conserved cysteines in the intracellular domain near the C-terminus. In contrast to Ctr1, Ctr4 of fission yeast *Schizosaccharomyces pombe* has two cysteine residues, Cys118 and Cys132, in the N-terminal extracellular domain in addition to several Mets motifs [7].

Another characteristic feature of the N-terminal domain of Ctr4 is a cluster of aromatic residues in between Cys118 and Cys132. These two cysteines are separated by 13 intervening residues including a palindromic sequence "YWNWY" rich in aromatic amino acids at its center. Therefore, the 15 amino acid residue sequence including the two cysteines, CKLSMYWNWYTIDAC, has a mirror-symmetrical arrangement of not only tryptophan and tyrosine but also cysteine residues. Aromatic amino acid residues, especially tryptophan, may potentially contribute to stabilization of Cu(I) based on a previous finding that metal recognition in a bacterial periplasmic CusF protein involves a cation–π interaction between Cu(I) and the aromatic ring of tryptophan together with coordinations by two methionines and a histidine [8].

In the present paper, we show that the cysteines in the N-terminal region of Ctr4 are bound to Cu(I) through the thiolate coordination in an air-saturated solution. The indole rings of the tryptophan residues that are present in between the two cysteines play a role in protecting the thiolate-Cu(I) center against oxidation via the π-electron-mediated interaction.

2. Materials and methods

A 24-mer peptide which contains the quasi-palindromic 15 amino acid sequence, CKLSMYWNWYTIDAC, in the N-terminal domain of Ctr4 (Ctr4NT) and its mutants were synthesized on an Applied Biosystems Model 431A by using Fmoc chemistry. The full sequences of the synthesized peptides are given in Table 1. The N-terminus of each peptide was acetylated. The peptide was deprotected and simultaneously cleaved from the resin by 7.5% (w/v) phenol in 2.5% 1,2-ethanedithiol, 5% thioanisole, 5% H_2O in trifluoroacetic acid. Crude products were precipitated with *tert*-butyl methyl ether, dissolved in H_2O and lyophilized. After pre-reduction treatment with dithiothreitol, the

* Corresponding author.
E-mail address: tmiura@m.tohoku.ac.jp (T. Miura).

http://dx.doi.org/10.1016/j.jinorgbio.2016.02.004
0162-0134/

Table 1
Amino acid sequences of Ctr4NT and its mutants[1].

Wild-type	KKDSSCKLSMYWNWYTIDACFKKK
WF	KKDSSCKLSMYFNFYTIDACFKKK
(W,Y)F	KKDSSCKLSMFFNFFTIDACFKKK
(W,Y)A	KKDSSCKLSMAANAATIDACFKKK

[1] Amino acid sequences outside of the Cys/Trp motif were partly modified to improve solubility of the peptide (underlined amino acids).

peptides were purified by HPLC on a reversed-phase column (Cosmosil 5C18-AR300) using a gradient mobile phase of water-acetonitrile (10–40%) containing 0.05% (v/v) trifluoroacetic acid and then lyophilized. Spectrophotometrical quantitative analysis of thiols by using Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid, Dojindo Laboratories) [9] was performed to confirm that the final peptide product was in the fully reduced form, and furthermore remained unoxidized even under air at least for 24 h in moderately acidic solution (pH 5.0) unless $CuCl_2$ was added.

The sample solutions for spectral measurements were prepared by dissolving lyophilized peptide in deionized water followed by mixing with $CuCl_2$ solution. The pH of peptide solution or peptide-$CuCl_2$ mixed solution was adjusted to 5.0, which is close to the optimum pH for growth of fission yeast *S. pombe* [10], by adding aliquots of aqueous solution of NaOH. The concentrations of the wild-type and the WF-mutant Ctr4NT were determined from the UV absorption intensity of tryptophan and tyrosine residues at 280 nm (ε_{280} = 13,980 for wild-type and 2980 for WF). The intensity of a phenylalanine band at 1012 cm^{-1} in a 488.0-nm excited Raman spectrum of peptide solution was measured for concentration determination of the (W,Y)F and (W,Y)A peptides by using the NO_3^- band at 1047 cm^{-1} as an internal intensity standard. Peptide concentration was adjusted to 50 μM for luminescence excitation and emission, 0.5 mM for visible absorption, 0.1 mM for UV absorption, 0.2 mM for UV resonance Raman (UVRR), and 5 mM for non-resonant Raman spectral measurements, respectively.

Luminescence excitation and emission spectra were collected on a Jasco FP-6500 spectrofluorometer. UV–visible absorption spectra were recorded on Hitachi U-3300 or U-3310 spectrophotometer with a quartz cell of 1-mm (UV) or 10-mm path length (visible). UVRR spectra were excited with 229-nm continuous wave radiation from an intracavity frequency-doubled Ar^+ ion laser (Coherent Innova 300 FReD) and recorded on a UV Raman spectrometer (Jasco TR-600UV) equipped with a CCD detector (Princeton Instruments LN/CCD-1752). Non-resonant Raman spectra were excited with the 488-nm line of a diode laser (Cyan-488-150, Spectra-Physics) and recorded on a micro-Raman spectrometer (Jasco NRS-3100) equipped with a thermoelectrically cooled CCD detector (DU401-BV-120, Andor). All spectral measurements were performed at 24 °C.

3. Results

3.1. Reduction of Cu(II) and stabilization of Cu(I) by the N-terminal domain of Ctr4

A complex formation between copper and Ctr4NT was examined by luminescence spectroscopy (Fig. 1). The 300-nm excitation emission spectra of Ctr4NT in the absence and presence of copper ions are shown in Fig. 1B. A distinct luminescence appears at around 550 nm immediately after addition of $CuCl_2$. This orange luminescence with large Stokes shift is known to be characteristic of the thiolate coordinated copper ion in the +1 oxidation state [11,12]. The 300-nm light excites the charge-transfer transitions between Cu(I) and thiolate ligands. The charge-transfer transitions are observed as broad bands around the L_b band of tryptophan at 280 nm in the absorption spectrum of Ctr4NT

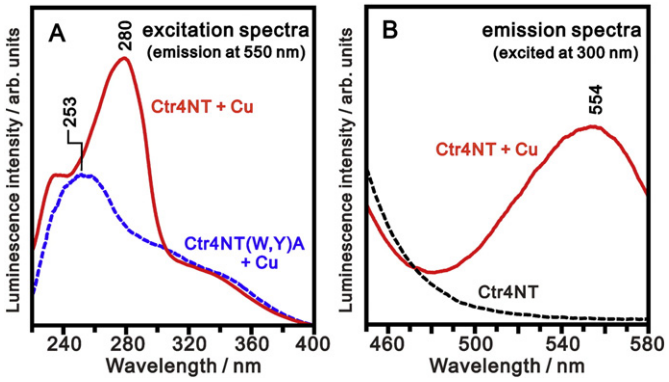

Fig. 1. Luminescence excitation and emission spectra of Ctr4NT after addition of $CuCl_2$ (peptide and copper concentrations of 0.05 mM, pH 5.0). (A) Excitation spectra (λ_{em} = 550 nm) of Ctr4NT and a mutant in which all tryptophan and tyrosine residues are replaced with alanine [(W,Y)A]. (B) Emission spectrum (λ_{ex} = 300 nm) of Ctr4NT and the control spectrum in the absence of copper.

after addition of $CuCl_2$ (Supplementary Fig. S1). Ctr4NT may be bound to Cu(I) via cysteine residues, even though the copper in the +2 oxidation state was added to the peptide under air-saturated conditions. This result suggests that Cu(II) is readily reduced to Cu(I) by Ctr4NT and then the Cu(I) is stabilized by the peptide through the thiolate coordination. The absence of the *d–d* transition band of Cu(II) in a visible absorption spectrum of Ctr4NT within a few hours after addition of $CuCl_2$ is also consistent with the copper binding in its reduced Cu(I) form with fully occupied *d* orbitals (see Section 3.2).

Two Cu(II) ions are expected to be reduced to Cu(I) upon formation of one disulfide bond, if the reduction of Cu(II) undergoes a one-electron transfer from thiolate to Cu(II) as suggested by previous studies [13]. Since Ctr4NT has two cysteines, the peptides with cysteine residues in the reduced form may still remain unoxidized after the redox reaction in the presence of equimolar copper to peptide. Therefore the reduced Cu(I) may be coordinated by thiolate ligands of unoxidized peptides. The coexistence of cysteine residues in the disulfide-bridged and metal-bound states can be confirmed by measuring Raman spectra of the peptide. Ctr4NT has cysteines in the SH form before addition Cu(II), which is confirmed by the appearance of the S–H stretching band at 2564 cm^{-1} in the Raman spectrum of metal-free peptide (Fig. 2A, lower trace). On addition of Cu(II) to the peptide, this band disappeared (Fig. 2A, upper trace), and concomitantly, the S–S stretching band appeared at 510 cm^{-1} (Fig. 2B, middle trace). Spontaneous air oxidation of cysteine residues is known to be negligible in metal-free solution over the pH range 3.0–7.5 even for a peptide with two cysteines [14]. Oxidation was also negligible for Ctr4NT before addition of $CuCl_2$ under our experimental condition (pH 5.0) as described in the experimental part (Section 2). Therefore, the disulfide bond formation that occurs immediately after addition of $CuCl_2$ is exclusively ascribed to the oxidation by Cu(II). On the other hand, the 510 cm^{-1} band is weaker than that of the fully oxidized peptide (Fig. 2B, top trace), indicating that the cysteine residues were only partly oxidized to cysteine on addition of Cu(II). Basically all the unoxidized cysteines are considered to be involved in the metal complex formation, because no cysteine residues remain to be in the SH form after addition of Cu(II) (Fig. 2A, upper trace). We have also confirmed by mass spectrometry that the oxidized Ctr4NT with a disulfide bond and the copper-bound Ctr4NT coexist soon after addition of Cu(II) (Supplementary Fig. S2). The stoichiometric details of the Cu(I)-Ctr4NT binding must await further investigation, because metal-dependent structural polymorphism of a flexible metal binding sequence makes such a stoichiometric analysis often complicated [15]. However, a 1:1 Cu(I)-Ctr4NT complex can be identified to be a main product, at least soon after the addition of equimolar or less equimolar Cu(II) to the peptide, according to the mass spectrometric data (Supplementary Fig. S2).