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Cu(I) binding properties of a designed metalloprotein

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ABSTRACT

The Cu(I) binding properties of the designed peptide C16C19-GGY are reported. This peptide was designed to form an α-helical coiled-coil but modified to incorporate a Cys-X-X-Cys metal-binding motif along its hydrophobic face. Absorption, emission, electrospray ionization mass spectrometry (ESI-MS), and circular dichroism (CD) experiments show that a 1:1 Cu-peptide complex is formed when Cu(I) is initially added to a solution of the monomeric peptide. This is consistent with our earlier study in which the emissive 1:1 complex was shown to exist as a peptide tetramer containing a tetranuclear copper cluster Kharenko et al. (2005) [11]. The presence of the tetranuclear copper center is now confirmed by ESI-MS which along with UV data show that this cluster is formed in a cooperative manner. However, spectroscopic titrations show that continued addition of Cu(I) results in the occupation of a second, lower affinity metal-binding site in the metallopeptide. This occupancy does not significantly affect the conformation of the metallopeptide but does result in a quenching of the 600 nm emission. It was further found that the exogenous reductant tris(2-carboxyethyl)phosphine (TCEP) can competitively inhibit the binding of Cu(I) to the low affinity site of the peptide, but does not interact with Cu(I) clusters.

1. Introduction

Metal-containing proteins comprise an important class of biological molecules which have evolved to perform such important functions as the storage and transfer of biological ligands, the enzymatic transformation of chemical substrates, and the facilitation of redox-dependent biological reactions [1]. This diversity of biochemical functions is due to the rich chemistry of transition metals and simple principles of inorganic coordination chemistry can often be used to understand the mechanisms of metalloprotein function. However, it is also known that the protein environment can play a significant role in fine-tuning the behavior of inorganic cofactors so that they can properly fulfill their biological function. Much effort has thus been devoted to gain a better understanding of the relationship between inorganic cofactors and their biological environments. To help advance this work, the field of metalloprotein design seeks to investigate ways in which metal-binding sites can be rationally engineered in peptides and proteins in order to produce new types of chemically-active species [2–5]. This work offers the possibility of creating non-native metalloproteins having tailorable chemical functions for application as technologically important materials [6–8].

It is within this context that our group has been studying the metal-binding properties of a cysteine-containing polypeptide called C16C19-GGY [9–12]. As previously reported by our group, the sequence of C16C19-GGY is based on a two-stranded α-helical coiled-coil [13,14,15] but modified to incorporate a Cys-X-X-Cys metal-binding motif along its hydrophobic face. It was found that while this change in sequence caused the apopeptide to exist as a disordered random coil, the addition of up to stoichiometric amounts of Cu(I) to the peptide solution produced a 1:1 metal-peptide complex consisting of single, tetrameric species in which extended X-ray absorption fine structure (EXAFS) studies suggested the presence of a multinuclear Cu(I) cluster [11]. It was also found that this metallopeptide displayed an emissive excited-state which can undergo collisional photoinduced electron-transfer in the inverted Marcus region [10]. It is noted that small molecule systems do not show such behavior since their high driving force reactions are rate-limited by diffusion [16]. These results show how the chemical properties of protein-bound metal ions can differ markedly from those in free solution.

As noted in our previous publication [11], our model of the 1:1 complex of Cu(I) and C16C19-GGY indicated that some of the peptide's available cysteine residues must remain uncomplexed to metal atoms. This suggested that further metal-binding interactions could occur and we thus sought to further characterize the metal-binding properties of this peptide. As will be presented below, it was found that higher Cu(I) loading into a lower affinity binding

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site can indeed occur, and that this occupancy does not change the peptide oligomerization state of the metallated complex. Electrospray ionization mass spectroscopy (ESI-MS) data give direct evidence for the presence of a Cu_4-species which can be converted into a Cu_5-species by further Cu addition which also quenches the emission of the 1:1 complex. It will further be shown that the exogenous reductant tris(2-carboxyethyl)phosphine (TCEP) alters the metal-binding properties of the low affinity site of the metallopeptide by acting as a competitive binder of Cu(I) but does not affect the spectroscopic properties of the cluster. This observation has relevance to studying the metal-binding properties of native proteins having relatively weak affinities for Cu(I).

2. Experimental section

2.1. Materials

The Fmoc-protected L-amino acid derivatives, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and anhydrous hydroxybenzotriazole (HOBT) were purchased from Peptides International Inc. (Louisville, KY). N-methylpyrrolidone (NMP) was purchased from Applied Biosystems Inc. (Carlsbad, CA). Piperidine, N,N-diisopropylethylamine, and tetrakis(acetonitrile)copper(I) hexafluorophosphate were purchased from Sigma–Aldrich (St. Louis, MO). All reagents were used as received without further purification.

2.2. General methods

Reverse-phase high performance liquid chromatography (HPLC) analyses were performed on either a preparative Vydac C-18 column (10 μM particle size, 22 × 250 mm) or a semipreparative Vydac C-18 column (10 μM particle size, 10 × 250 mm). A two-pump system (Waters Model 515) equipped with a Waters Model 996 diode array detector/spectrophotometer having a 1-cm path length cell was used. Peptide purification was effected using a linear gradient of acetonitrile and water both containing 0.1% (v/v) trifluoroacetic acid as previously described [13].

2.3. Peptide synthesis

The peptide C16C19-GGY has the sequence Ac-K-$(IEALEGK)_2$-(CEACEGK)-(IEALEGK)-GGY-amide, and was synthesized on an Applied Biosystems Model 433 A peptide synthesizer using standard Fmoc-chemistry with the manufacturer's FMOC amide resin. The 0.25 mmol scale protocol with a N-terminal capping strategy by acetic anhydride was used. Activation was achieved by HBTU and HOBT in *N,N*-dimethylformamide. Deprotection of the amino acid side chains and cleavage from the resin were performed by reaction with a mixture of trifluroacetic acid (82.5% v/v), phenol (5% v/v), dithiothreitol (DTT) (2.5% v/v), thioanisole (5% v/v) and water (5% v/v) for 2.5 h at room temperature. The crude peptide was then precipitated in cold anhydrous diethyl ether, collected by vacuum filtration and dried under vacuum. Final purification was achieved by preparative reversed-phase C18 HPLC.

2.4. UV spectroscopy

The UV–visible (UV–vis) absorption spectra were recorded on a Hewlett–Packard model 8452A diode array spectrophotometer. The molar extinction coefficient, $\varepsilon(275\ \text{nm}) = 1405\ M^{-1}\ cm^{-1}$ [17], was used to determine the concentration of the apopeptide which has a single tyrosine residue. Peptide samples (100–200 μM) were prepared in 0.1 M acetate buffer, pH 5.5, in a 1 cm pathlength cell. Care was taken to avoid the presence of Cu(II) in all titration experiments. Stock solutions of Cu(I) (0.01 M) were made by dissolving tetrakis(acetonitrile)copper(I) hexafluorophosphate in argon purged acetonitrile in which Cu(II) remains insoluble, and additions of Cu(I) to solutions of the peptide were performed in a Coy anaerobic chamber. For each addition of Cu(I), 2–5 min were allowed for the system to reach equilibrium before taking the UV measurement.

2.5. Circular dichroism spectroscopy

Circular dichroism spectra were obtained on an Aviv model 202-01DS circular dichroism spectrometer (Lakewood, NJ). Samples were measured in 0.1 M acetate buffer, pH 5.5 using a rectangular 0.1 cm or 0.5 cm pathlength cell. The spectra were obtained as an average of 3–5 scans using 1 nm steps. Mean residue molar ellipticities were calculated according to the equation:

$$[\Theta] = [\Theta]_{\text{obs}}/(10lcn)$$

where $[\Theta]_{\text{obs}}$ is the observed ellipticity measured in millidegrees, l is the path length of the cell in centimeters, c is the molar concentration of the peptides, and n is the number of amino acid residues in the peptides. Titration experiments were performed as described above.

2.6. Steady-state luminescence spectroscopy

Steady-state luminescence spectra were obtained using a QM4SE fluorometer from Photon Technology International Inc. (London, ON). Samples were excited at either 300 nm or 355 nm and a 400 nm long-pass filter was used when recording the spectra. Titration experiments were performed as described above.

2.7. High performance size exclusion chromatography (HPSEC)

HPSEC experiments were performed on a Superdex 75 10/300 GL column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) [17] having an optimum molecular weight range of 3–70 kDa for globular proteins. The column was connected to a Waters Model 515 high performance liquid chromatography system equipped with Waters Model 996 diode array detector. Samples were eluted using a 50 mM KH_2PO_4/100 mM KCl buffer, pH 7.0, with a flow rate of 0.3–0.4 ml/min and monitored at 230 nm.

2.8. Electrospray ionization mass spectrometry (ESI-MS) titration

Lyophilized samples of C16C19-GGY were incubated in 490 mM TCEP for 1 h at room temperature followed by desalting on a Sephadex G-25 (Amersham Bio-sciences, Uppsala Sweden) column with 5 mM ammonium formate pH 5.7 (J.T. Baker, Phillipsburg, New Jersey). Protein solutions were argon saturated and rigorously evacuated. Cu(I) stock solutions were prepared as described above. Data were measured using an electrospray ionization-liquid chromatography-time of flight (ESI-LC-TOF) mass spectrometer (Waters Micromass Inc., Milford Massachusetts) in the positive ion mode. NaI was used as the calibrant. The scan conditions for the spectrometer were: capillary, 3000 V; sample cone, 15 V; extraction cone, 10 V; radio frequency lens, 450 V; desolvation temperature, 20.0 °C; source temperature, 80.0 °C; cone gas flow, 41 l h^{-1}; and desolvation gas flow, 445 l h^{-1}. The m/z range was 500.0–3000.0; the scan mode was continuum, with a scan time of 2.4 s and an interscan delay of 0.10 s. Spectra were constructed and deconvoluted using the MASSLYNX v.4.0 software package.