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Copper·Lys-Gly-His-Lys mediated cleavage of tRNA^{Phe}: Studies of reaction mechanism and cleavage specificity

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ABSTRACT

The reactivity of $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$ and $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^{+}$ toward tRNA^{Phe} has been evaluated. The amidated and carboxylate forms of the copper peptides display complex binding behavior with strong and weak sites evident ($K_{D1}^{app} \sim 71\ \mu M$, $K_{D2}^{app} \sim 211\ \mu M$ for the amide form; and $K_{D1}^{app} \sim 34\ \mu M$, $K_{D2}^{app} \sim 240\ \mu M$ for the carboxylate form), while Cu^{2+}(aq) yielded $K_{D1}^{app} \sim 81\ \mu M$ and $K_{D2}^{app} \sim 136\ \mu M$. The time-dependence of the reaction of $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^{+}$ and $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$ with tRNA^{Phe} yielded $k_{obs} \sim 0.075\ h^{-1}$ for both complexes. HPLC analysis of the reaction products demonstrated guanine as the sole base product. Mass spectrometric data shows a limited number of cleavage fragments with product peak masses consistent with chemistry occurring at a discrete site defined by the structurally contiguous D and TΨC loops, and in a domain where high affinity magnesium centers have previously been observed to promote hydrolysis of the tRNA^{Phe} backbone. This cleavage pattern is more selective than that previously observed by Long and coworkers for nickel complexes of a series of C-terminally amidated peptides (Gly-Gly-His, Lys-Gly-His, and Arg-Gly-His), and may reflect variations in structural recognition and a distinct reaction path by the nickel derivatives. The data emphasizes the optimal positioning of the metal-associated reactive oxygen species, relative to scissile bonds, as a major criterion for development of efficient catalytic nucleases or therapeutics.

1. Introduction

There has been significant interest in understanding the interaction of metal complexes with DNA, providing insight on the molecular mechanisms by which chemistry with DNA can occur [1–4]. Such interest is fueled by a desire to develop novel drug molecules [5–8], artificial nuclease and crosslinking reagents [9], and in fundamental investigations of DNA recognition. Recent advances in the understanding of the structural and functional complexity of RNA have led to similar interest in the reaction chemistry and structural mechanisms underlying recognition of complex RNA motifs with metal ion complexes. The structural complexity of RNA, coupled with the functional activity that is often essential for the survival of pathogenic organisms and viruses [10,11], has spurred renewed interest in RNA from both a fundamental perspective and for potential applications in medicine [12,13], genetic regulation [14], and other areas of cellular chemistry [15,16]. Metal-promoted scission of structured RNA targets is a developing area of metallodrug design [17]. Important for the development of such drugs is a detailed understanding of the mechanism for metal-promoted cleavage of RNA, especially when mediated by oxidative damage. While the pathways for oxidative degradation of DNA are well established [18], the influence of RNA structure and the unique presence of 2′–OH functionality on reaction mechanism are not clearly defined [19].

The amino terminal copper and nickel binding (ATCUN) motif derived from the N-terminus of serum albumins [20] has been shown to bind to a variety of metals with a dissociation constant for divalent copper ion on the order of $10^{-16}\ M^{-1}$. Long and coworkers have examined the interaction of Ni(ATCUN) complexes with RNA and observed cleavage only under conditions that included the use of oxone and treatment with aniline acetate [21]. In this report the cleavage of tRNA^{Phe} by Cu(ATCUN) complexes in the presence of ascorbate and dioxygen is studied in detail and is compared with findings for related nickel ATCUN complexes. The influence of amidation of the C-terminus of the ATCUN motif on both binding and chemistry is reported.

2. Materials and methods

2.1. Materials

Yeast tRNA^{Phe} was purchased from Sigma. ATCUN motifs were purchased from Bachem (CA), or from Genemed Synthesis Inc. (South San Francisco, CA).

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Table 1
Solvent elution profile used for HPLC analysis.

Time (min)	0	10	25	40	50	60	74
% Acetonitrile	0	0	0.6	2.0	10.0	30.0	Stopped

2.2. HPLC analysis

Samples were loaded onto a VYDAC monomeric C-18 reverse phase column equilibrated with 0.1 M ammonium acetate (NH_4OAc), pH = 6.8 with a flow rate of 0.500 mL/min. The products were eluted with acetonitrile according to the gradient described in Table 1 and monitored at 254 nm.

The column was calibrated for the elution of adenine, guanine, uracil, and cytosine, as well as the two copper-ATCUN complexes used in these experiments, $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^+$ carrying a free C-terminal carboxyl, and $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$ with C-terminal amidation. To monitor base release from the cleavage of $tRNA^{Phe}$, reaction samples containing $tRNA^{Phe}$, copper-ATCUN complex, and ascorbate (5 mM) in 50 mM Tris buffer, pH = 7.4 were loaded onto the HPLC column following incubation at various times.

2.3. Reaction kinetics

The kinetics of metallopeptide-promoted scission of $tRNA^{Phe}$ was followed by monitoring the rate of disappearance of $tRNA^{Phe}$ substrate for each cleavage reaction. The working solution contains $tRNA^{Phe}$ (200 μM), copper-ATCUN complex (200 μM), $MgCl_2$ (10 mM), and ascorbate (5 mM) in 50 mM Tris buffer (pH = 7.4). EDTA (5 mM) was added to stop the reaction, as well as urea (6 mM), which were added immediately before loading and denatured by heating at 90 °C for 5 min. Residual $tRNA^{Phe}$ was determined by calculating the area of the peak for $tRNA^{Phe}$ following reaction. The same amount of EDTA and urea were added to controls according to the same procedure.

2.4. Binding experiments

The binding affinities of $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^+$ and $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$ toward $tRNA^{Phe}$ were determined fluorimetrically by following the excitation of bases on a Perkin Elmer LS 50B Luminescence Spectrometer. The resulting binding curves were fit bys use of a standard Equation (1) that accommodates two distinct classes of binding site (strong and weak) to yield apparent binding constants (K_{D1} and K_{D2}) using Origin 7.0 software, where I is the observed emission intensity at the concentration of binding species C (copper ion or copper complexes), I_B is the emission intensity in the fully bound state, I_F is the emission intensity in the fully free state, and I_I is the emission for the intermediate bound state:

$$I = (K_{D1}\cdot K_{D2}\cdot I_F + K_{D2}\cdot C\cdot I_I + C^2\cdot I_B)/(K_{D1}\cdot K_{D2} + K_{D1}\cdot K_{D2}\cdot C + K_{D1}\cdot K_{D2}\cdot C^2)]I_B \quad (1)$$

A working solution containing $tRNA^{Phe}$ (20 nM) in 50 mM Tris buffer (pH = 7.4) and 10 mM $MgCl_2$ was prepared and placed in a quartz cuvette. Fluorescence was monitored using excitation and emission wavelengths of 260 nm and 520 nm, respectively and a slit width of 4 nm. The influence of serial addition of aliquots of copper peptide or copper ion on emission intensity was recorded, and no correction was required for inner filter effects at the wavelengths used.

2.5. Mass spectrometry

Reactions for mass spectrometry were carried out in 40 μL reaction volumes with the following concentrations: 100 μM $tRNA^{Phe}$, 50 mM Tris buffer (pH = 7.4), 10 mM $MgCl_2$, 100 μM copper·ATCUN complex, 5 mM ascorbate. Reactions were run for 24 h and then submitted for mass spectrometric analysis. The samples were dialyzed and desalted using a ZipTip (Millipore, Bedford, MA) prior to analysis via matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. MALDI experiments were performed on a Bruker Reflex III (Bruker, Breman, Germany) mass spectrometer operated in linear, positive ion mode with a N_2 laser. Laser power was used at the threshold level required to generate signal. The accelerating voltage was set to 28 kV.

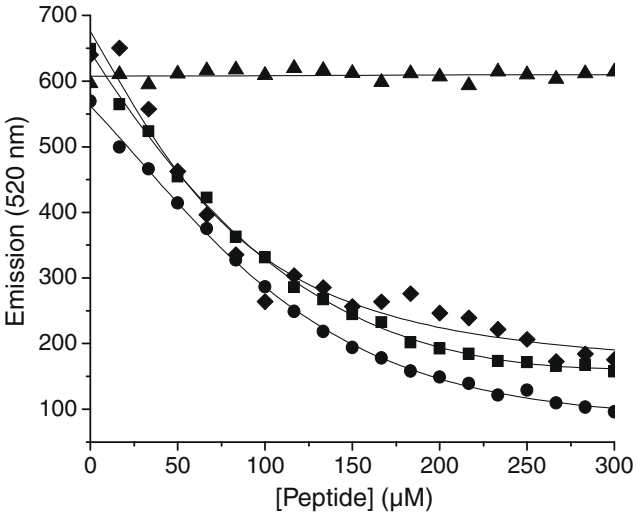

Fig. 1. Binding curves for $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^+$, $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$, Cu^{2+}(aq), and the metal-free peptide are shown. Triangles = Lys-Gly-His-Lys, circles = $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$, squares = $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^+$, and diamonds = Cu^{2+}(aq). The observed emission intensity at 520 nm was fit to the concentration of binding species (copper peptide or free copper ion) by use of Eq. (1), which yielded apparent values for K_{D1} and K_{D2} for $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^+$, $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$, and Cu^{2+}(aq).

3. Results

3.1. Binding of Cu^{2+}·ATCUN to $tRNA^{Phe}$

Binding of $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^+$ and $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$ to $tRNA^{Phe}$ is shown in Fig. 1. The RNA was not labeled with a fluorophore, but binding was monitored by following the intrinsic fluorescence of the bases. It is clear that both complexes bind similarly, but that the peptide control (no copper) does not show significant binding under these solution conditions. Optimal fitting of both copper peptides and free Cu^{2+}(aq) required a weaker and higher affinity site. The amidated $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$ complex showed apparent affinities as $K_{D1}^{app} \sim 71$ μM and $K_{D2}^{app} \sim 211$ μM, while the carboxylated form showed $K_{D1}^{app} \sim 34$ μM and $K_{D2}^{app} \sim 240$ μM. Binding of $CuCl_2$ to the $tRNA^{Phe}$ under the same conditions was also observed, yielding $K_{D1}^{app} \sim 81$ μM and $K_{D2}^{app} \sim 136$ μM.

3.2. Cu^{2+}·ATCUN mediated cleavage of $tRNA^{Phe}$ and HPLC characterization of reaction product

The time-dependence of the reaction of $[Cu^{2+}\cdot$Lys-Gly-His-Lys$]^+$ and $[Cu^{2+}\cdot$Lys-Gly-His-Lys-$NH_2]^{2+}$ with $tRNA^{Phe}$ was monitored by HPLC and the disappearance of the RNA peak over time was used to monitor the kinetics of the reaction. By monitoring the loss of peak intensity as a function of time, a $k_{obs} \sim 0.075\ h^{-1}$ was determined for both complexes. No activity was observed for free copper ion under comparable reaction conditions and concentrations of tRNA and catalyst.