Targeted Cleavage of HIV Rev Response Element RNA by MetalloPeptide Complexes

Yan Jin and J. A. Cowan*

Evans Laboratory of Chemistry, Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210

SUPPORTING MATERIAL

1. MATERIALS & METHODS

1.1 General materials. The peptides used in this study were purchased from Genemed Synthesis Inc (South San Francisco, CA). RRE RNA, with or without 5’ fluorescein labeling, was purchased from Dharmacon RNA Technologies (Lafayette, CO). All materials were prepared under RNase free conditions as previously described1 or purchased in the highest commercially available grades. Unless stated, all other reagents used in this research were obtained from Sigma chemical Co.

1.2 Synthesis and characterization. A solution of 2.2 mM peptide in 50 mM Hepes buffer (pH = 7.4) was mixed with 2 mM CuCl₂ in 50 mM Hepes buffer solution in 1:1 v:v ratio. This yielded a 1.1:1 peptide to copper ratio, and so there was no free Cu²⁺ (aq) ion in the cleavage reaction mixture. The resulting solution was mixed at room temperature for ~ 30 min, resulting in a stable light reddish-purple solution that displayed a UV-vis spectrum typical of Cu-ATCUN complexes.² Samples were maintained at 4 °C and concentrations and stabilities of the Cu-peptide complexes were routinely checked prior to use.

1.3 Fluorescence spectroscopy. A 300 µL volume of a 6 nM solution of 5’-fluorescein labeled RRE RNA (molecular concentration) was prepared in 20 mM Hepes buffer, 100 mM NaCl, pH = 7.4, and the fluorescence response before and after addition of a 1 µl aliquot of 5 µM peptide or Cu-peptide complex was measured in a quartz cuvette at room temperature using a Perkin Elmer LS50B spectrofluorimeter, with excitation (SW = 6 nm) and emission (SW = 6 nm) wavelengths set at 490 nm and 515 nm, respectively. Dissociation constants were obtained by fitting fluorescence quenching date to a one site binding equation \( r = \frac{B \cdot [\text{pep}]}{K_d + [\text{pep}]} \) with binding function \( r = \frac{(y_t - y_0)}{(y_{\text{final}} - y_{\text{initial}})} \) versus [pep] is the concentration of metal-free or metal-bound peptide (Figure SM1). Each point was corrected for the dilution effect. Since our Cu-Rev1 complex has very little absorbance (\( \varepsilon_{515} \sim 30 \text{ M}^{-1}\text{cm}^{-1} \)), the inner filter effect³ is negligible in this assay.

1.4 RNA cleavage studies with gel electrophoresis. In general, RNA cleavage reactions were performed in 5 µl total volumes with 10 µM 5’-fluorescein labeled RRE RNA (molecular concentration), various concentrations of Cu-peptide complexes, and with or without added ascorbate in 20 mM Hepes buffer, 100 mM NaCl, pH = 7.4 at 37 °C. Control reactions were carried out at the same time (Figures SM2 to SM4). The reactions were quenched with a loading buffer containing 8 M Urea and 0.5 M EDTA, but without dyes.¹ The loading buffer
with dyes was run in another lane. The reaction samples were loaded onto either 15% or 20% polyacrylamide/8M urea denaturing gels (from American Bioanalytical) and electrophoresed at 300 V for 5-8 h. To avoid degradation or photobleaching of fluorescein, the reactions and gel electrophoresis were performed in the dark. Each experiment was carried out at least in triplicate.

1.5 Mass spectrometric characterization of RNA cleavage products. RNA cleavage reactions were performed in 40 µl total volumes with 100 µM unlabeled RRE RNA, 1:1 concentration ratio of Cu-peptide complex with, 1 mM ascorbate in 20 mM Hepes buffer, 100 mM NaCl, pH = 7.4 at 37 °C. Control reactions were carried out with RRE RNA and ascorbate. ESI/MS was used to get the mass of the cleavage products. Electrospray ionization (ESI) experiments were performed on a Micromass Q-Tof(tm) II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal nanospray source from New Objective, Inc. (Woburn, MA) operated in negative ion mode. Sodium Iodide was used for mass calibration for a calibration range of m/z 500 - 3000. Salt buffers from the RNA samples were removed using ZipTips (Millipore, Billerica, MA) following recommended manufacturer protocols. The elutants from the ZipTip were used directly and infused into the electrospray source at a rate of 2 ml.min⁻¹. Optimal ESI conditions were: capillary voltage 3000 V, source temperature 110 °C and a cone voltage of 60 V. Q1 was set to optimally pass ions from m/z 500 – 3000 and all ions transmitted into the pusher region of the TOF analyzer were scanned over this m/z range with a 1 s integration time. Data was acquired in continuum mode until acceptable averaged data was obtained (10 -15 minutes). ESI data was deconvoluted using MaxEnt I provided by Micromass (Figures SM5 to SM7) and the cleavage sites were assigned (Figure SM8, Scheme SM1, Table SM1). It is general practice to analyze the products of an RNA cleavage reaction by mass spectrometry where the reaction mixture contains at least 2 nmole of substrate,⁴-⁶ since it is technically difficult to extract the RNA products from a gel and then do the analysis. (see for example Meng, M.; Limbach, P. A. Int. J. Mass Spectrom. 2004, 234, 37-44.; Meng, M.; Limbach, P. A. Anal. Chem. 2005, 77, 1891-1895.; De Iuliis, G. N.; Lawrance, G. A.; Wilson, N. L. Inorg. React. Mech. 2002, 4, 169-186).
Figure SM1. Determination of $K_D$'s for complex formation of RRE RNA with Rev1 and Cu-Rev1. The $K_D$ for RRE binding to Rev1 is $\sim 30$ nM (a), while $K_D$ for RRE binding to Cu-Rev1 is $\sim 35$ nM (b). These numbers are consistent with the $K_D$ measured for RRE RNA and native Rev peptide lacking the ATCUN motif ($\sim 40$ nM), consistent with the absence of any significant influence of the N-terminal GGH sequence, or its copper complex, on binding to the RRE RNA.
**Figure SM2.** (left) RNA cleavage of 5' FL labeled RRE, R23, and HCV IRES RNA by Cu$^{2+}$-Rev1 peptide complex in the presence of O$_2$ and ascorbate. Lanes: 1. RRE (10 µM) + Cu$^{2+}$-Rev1 peptide complex (10 µM) + ascorbate (100 µM); 2. RRE RNA (10 µM) + ascorbate (100 µM); 3. R23 (10 µM) + Cu$^{2+}$-Rev1 peptide complex (10 µM) + ascorbate (100 µM); 4. R23 RNA (10 µM) + ascorbate (100 µM); 5. HCV IRES RNA (10 µM) + Cu$^{2+}$-Rev1 peptide complex (10 µM) + ascorbate (100 µM); 6. HCV IRES RNA (10 µM) + ascorbate (100 µM); Reactions were incubated in 20 mM Hepes buffer, 100 mM NaCl, pH = 7.4, for 3 hours at 37 °C. Reaction products were separated by 15% denaturing PAGE. Some impurities (*) in the HCV IRES sample are noted. (middle) a schematic illustration of the stem loop structure adopted by R23,$^8$ and (right) a schematic illustration of the secondary structure of the internal ribosome entry site (IRES) RNA of Hepatitis C Virus (HCV).$^9$
Figure SM3. RNA cleavage of 5’ FL labeled RRE RNA by Cu$^{2+}$-Rev1 peptide complex or Cu$^{2+}$-KGHK in the presence of O$_2$ and ascorbate. Lanes: 1. RRE RNA (10 µM) + ascorbate (100 µM); 2, RRE (10 µM) + Cu$^{2+}$-Rev1 peptide complex (10 µM) + ascorbate (100 µM); 3. RRE (10 µM) + Cu$^{2+}$-KGHK complex (10 µM) + ascorbate (100 µM). Reactions were incubated in 20 mM Hepes buffer, 100 mM NaCl, pH = 7.4, for 3 hours at 37 °C. Reaction products were separated by 20% denaturing PAGE.
Figure SM4. RNA cleavage of 5’ FL labeled RRE RNA by Cu\(^{2+}\) - Rev1 peptide complex in the presence of O\(_2\) and ascorbate. Lanes: 1. RRE RNA (10 µM); 2, 3 RRE (10 µM) + Cu\(^{2+}\) - Rev1 peptide complex (10 µM) + ascorbate (100 µM); 4. RRE RNA (10 µM) + ascorbate (100 µM); 5. RRE RNA (10 µM) + Rev1 peptide (10 µM); 6. RRE RNA (10 µM) + Cu\(^{2+}\) (aq) (10 µM) 7. RRE (10 µM) + Cu\(^{2+}\) (aq) (10 µM) + ascorbate (100 µM). Reactions were incubated in 20 mM Heps buffer, 100 mM NaCl, pH = 7.4, for 3 hours at 37 °C. Reaction products were separated by 20% denaturing PAGE.
Figure SM5. Control reaction for RRE RNA cleavage analyzed by mass spectrometry. The control reaction was performed under similar solution conditions, but without the Cu-peptide complex.
Figure SM6. Control reaction for RRE RNA cleavage showing the accurate mass for substrate RRE RNA following deconvolution.
**Figure SM7.** Mass spectrometric analysis of the product mixture following the RRE cleavage reaction performed as described in Materials and Methods.

**Figure SM8.** NMR characterized tertiary structures of RRE RNA and Rev peptide,\(^\text{10}\) showing the binding pocket and cleavage sites determined from mass spectrometric analysis.
Scheme SM1. Proposed mechanism of RRE RNA cleavage by the Cu$^{2+}$-Rev1 complex with ascorbate under aerobic conditions (paths 1, 2, 3 leading to products 1, 2, and 3, as defined in Table SM1).
Table SM1. ESI-MS analysis of the cleavage products following treatment of RRE by the Cu$^{2+}$-Rev1 complex with ascorbate.$^a$

<table>
<thead>
<tr>
<th>product band on gel</th>
<th>sequence assignment</th>
<th>found m/z for hydrolytic cleavage</th>
<th>predicted m/z for hydrolytic cleavage</th>
<th>predicted m/z for oxidative cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-GGUC-Pi-X$^b$-3'</td>
<td>1425.3$^c$</td>
<td>1318.7$^c$</td>
<td>1425.7$^c,e$</td>
</tr>
<tr>
<td>2</td>
<td>5'-GGUCU-Pi-X$^b$-3'</td>
<td>1718.6$^c$</td>
<td>1624.9$^c$</td>
<td>1720.9$^c,f$</td>
</tr>
<tr>
<td>3</td>
<td>5'-GGUCUGGG-Pi-X$^b$-3'</td>
<td>1428.8$^d$</td>
<td>1329.8$^d$</td>
<td>1428.4$^d,g$</td>
</tr>
</tbody>
</table>

$^a$ Predicted mass values are based on atomic weight as (M+H)$^+$ unless specially stated. Cleavage products are determined from Fig SM7. $^b$ X stands for the partial ribose left after cleavage. In the case of hydrolytic cleavage, mass of X is zero. $^c$ z is -1. $^d$ z is -2. $^e$ Cleavage product through C-1'H abstraction with X: CH$_2$CH$_2$COCHO (through path 1 in Scheme SM1).$^{11}$ Mass of (M + Na)$^-$ was calculated. $^f$ Cleavage product through C-4'H abstraction (O$_2$) with X: CH$_2$COO$^-$ (through path 2 in Scheme SM1).$^{12,13}$ Mass of (M + K)$^+$ was calculated. $^g$ Cleavage product through C-4'H abstraction (H$_2$O) with X: CH$_2$COCH$_2$OH CHO (through path 3 in Scheme SM1).$^{14}$ Mass of (M + 2Na + K)$^{2-}$ was calculated.

SM References