Site-Specific Cleavage of the HIV-1 TAR RNA by a Hydroxysalen–Copper(III) Complex

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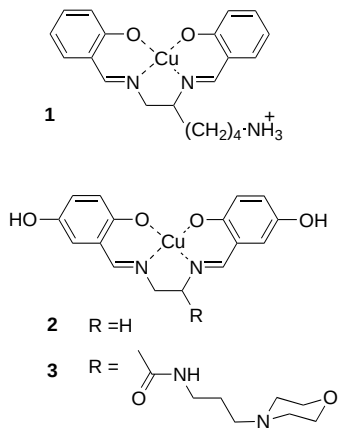
KEYWORDS:

copper · EXAFS spectroscopy · metal complexes · RNA · XANES spectroscopy



Metal complexes of *N*,*N*′-bis(salicylaldehydo)ethylenediamine, usually referred to as salens, represent a powerful group of versatile catalysts for the oxidation of polymers.[1] These tetradentate Schiff bases are routinely used for the epoxidation of olefins. Over the past decade, the applications of these molecules have greatly expanded and they are now used in biological studies, in particular as probes to study nucleic acids chemistry. Different series of salen–metal complexes have been designed to induce DNA damages and include Ni, Co, Cr, Fe, Mn, Ru, and Cu complexes, which can all be used as DNA cleaving agents under appropriate activation conditions.[2] We have shown previously that the functionalized salen–Cu^{II} complex **1** binds to DNA and induces strand scission in the presence of mercaptopropionic acid (MPA).[3] A self-activated nuclease was designed by equipping the salen **1** with hydroxy groups in the *ortho*, *meta*, or *para* position of each of the two salicylidene units, which provided complexes capable of cleaving DNA in the absence of activating agents.[4] The *para* isomer **2** was found to be particularly efficient at promotion of DNA cleavage. The mechanism proposed to explain the DNase activity of the nuclease involves the formation of an oxidizing Cu^{III} species. On the basis of electrochemistry and electron spin resonance experiments we reasoned that the hydroquinone system can cooperate with the redox metal system to trigger DNA cleavage.[4] In an extension of this work, we now present further spectroscopic evidences that **2** forms a stable complex with Cu^{III}. In addition, we show that this complex can also function as a powerful ribonuclease to induce site-specific cleavage of the hairpin-loop trans-activation-responsive region (TAR) RNA from HIV-1.

X-ray absorption near-edge structure (XANES) spectra of **2** at the copper K edge were recorded for the solid state and an ethanol solution of the complex. A transition was observed at 8981.2 eV only with the solid state species and the main edge was shifted by 2.3 eV (from 8996.6 to 8998.9 eV) for the solution complex compared to the solid-state species. This characteristic energy shift reflects the formation of Cu^{III} redox state species.[5]

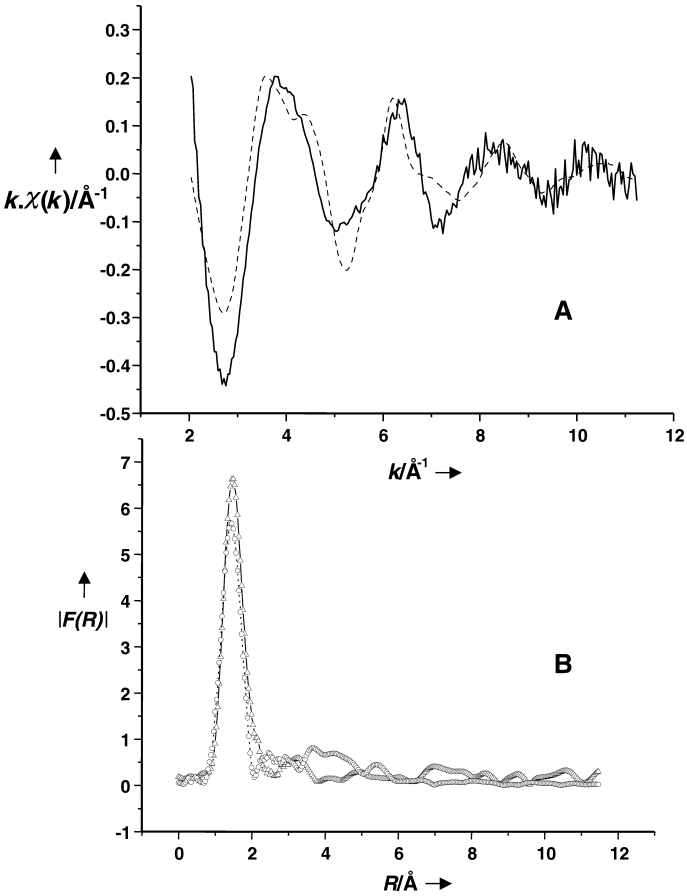


Figure 1. *A) Normalized EXAFS spectra of **2** in (solid line) the solid state and (dashed line) in solution (1 mM in ethanol). B) △ = solid state, ○ = in solution.*

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The Cu^{II} complex in the solid state is oxidized to a Cu^{III} species in the presence of molecular oxygen in the ethanol solution, in agreement with our previous hypothesis.[4] Experimental and Fourier-transformed extended X-ray absorption fine structure (EXAFS) spectra of **2** in the solid and liquid states are shown in Figure 1. In both cases, the first shell copper coordination was fitted satisfactorily with a single backscattering. Multiple backscattering was negligible within a distance of 0 – 3 Å from the first nitrogen/oxygen shell coordination. The oxygen and nitrogen phases and amplitudes were computed (with the FEFF ab initio program) by using Cu – O and Cu – N distances obtained from the crystallographic data of a Cu – salen complex.[6] The first shell of copper coordination with nitrogen and oxygen atoms for the complex in the solid state is in perfect agreement with the published X-ray data. Cu – O and Cu – N distances of 1.92 and 1.96 Å, respectively, were calculated. The analysis of the first coordination shells indicates the presence of an additional "neighbor" for the complex in solution. The simulation suggests that this neighbor is an additional oxygen atom ligated to the copper (at 1.89 Å), which forms a potential superoxo species.

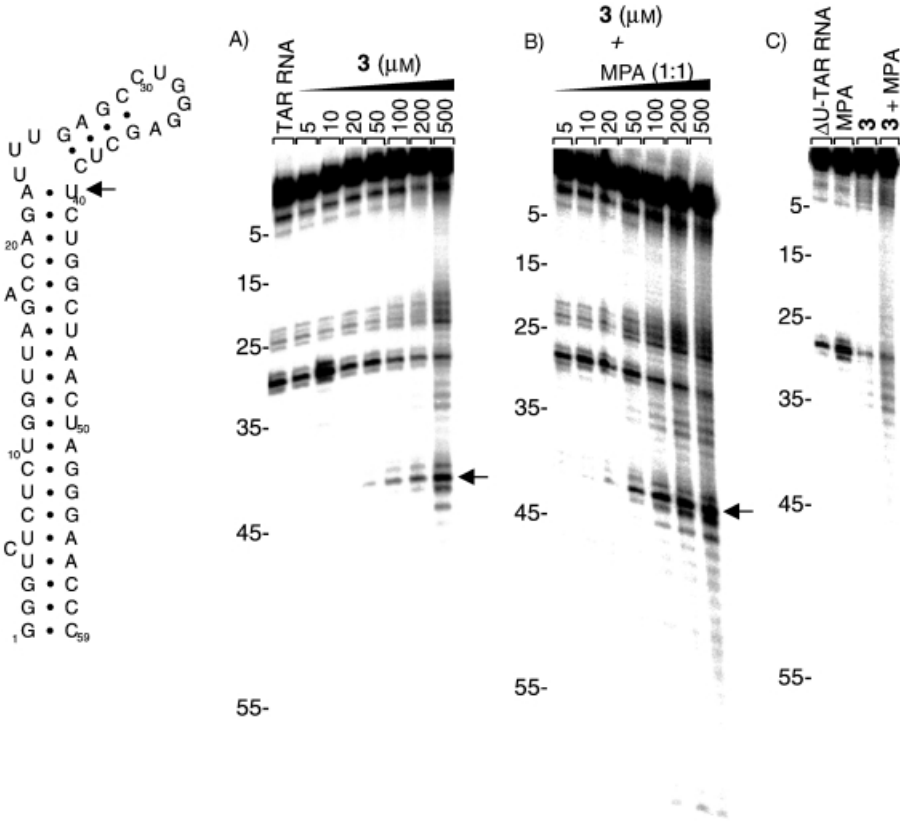

Figure 2. *Cleavage of the TAR RNA by **3** (A) in the absence and (B) in the presence of an equimolar amount of MPA. C) Cleavage of the ΔU-TAR RNA mutant, which lacks the uridine bulge. The control track labeled "(ΔU)TAR RNA" contained no metal complex. The secondary structure of the wild-type TAR RNA is shown with an arrow pointing to the main cleavage site at U40.*

The TAR RNA of HIV-1 was used as a substrate to evaluate the capacity of the hydroxysalen – Cu complexes to cleave RNA. This 60-nt RNA sequence, which is located at the 5′-end of all nascent HIV-1 transcript, adopts a hairpin secondary structure consisting of a highly conservative 6-nt loop and a 3-nt pyrimidine bulge flanked by 2 double-stranded stems (Figure 2). The cleavage experiments were initially performed with the unsubstituted complex **2**, which is sparingly soluble in water. To increase solubility in aqueous buffers and thus facilitate the handling of the metal complex for the biochemical experiments, we synthesized complex **3**, which bears a morpholino side chain. No difference was observed between **2** and **3** in terms of RNA cleavage and subsequent experiments were performed with **3** to limit the technical difficulties caused by the precipitation of the metal complex. Interestingly, significant RNA cleavages were detected in the absence of the reductant when relatively high concentrations (100 μM) of **3** were used (Figure 2 A). A specific and well-resolved cleavage site was detected at the uridine residue at position 40, located opposite the UUU bulge. A much weaker and more diffuse set of cleavage sites was also identified around position 24 in the UUU bulge region. Some background RNA damage at U31 caused by the fragility of the apical loop is also evident, even in the absence of the metal complex. This hypersensitive site is rendered less susceptible to cleavage in the presence of the metal complex, perhaps because of a ligand-induced conformational change of the RNA. No specific cleavage site was observed in the presence of free copper or with the metal-free salen derivative. When MPA was added, **3** exhibited significantly more cleavage activity and the band corresponding to U40 could be detected at lower concentrations (20 – 50 μM; Figure 3 a). The cleavage around U24 was also promoted by MPA. The extent of RNA cleavage at U40 is most pronounced with the *para* isomer **3**. The cleavage was almost insignificant with the corresponding *meta* isomer whereas the *ortho* isomer showed an intermediate behavior, with a cleavage capacity about 30 % inferior to that of the *para* isomer **3** (Figure 3 b). In terms of cleavage efficacy, the results obtained with the TAR RNA parallel those previously reported with DNA.[4]

The results suggest that the uridine bulge plays an important role in the cleavage of the RNA by **3**. To verify this idea, we produced a 57-nt ΔU-TAR RNA analogue that lacks the pyrimidine bulge. The deletion of the UUU bulge completely prevents the cleavage of the RNA by the metal complex (Figure 2 C). Even at a high concentration (500 μM), **3** has absolutely no effect on the integrity of ΔU-TAR RNA and the addition of the reductant does not restore the cleavage site at U40 seen with the wild-type RNA. Therefore, the pyrimidine bulge that separates the two duplex regions in the RNA is

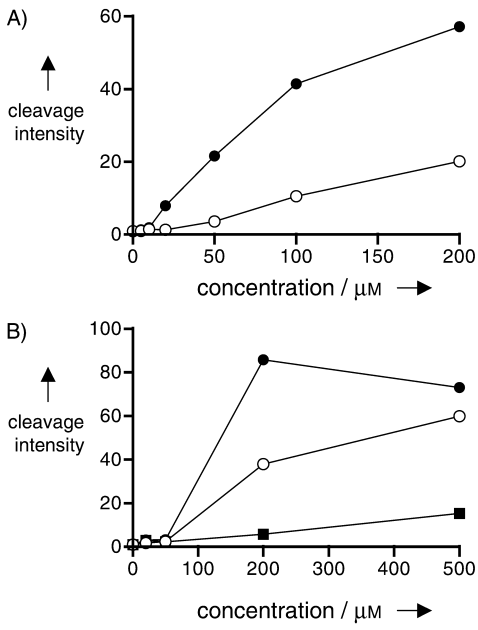

Figure 3. *Extent of RNA cleavage at the U40 reactive site within TAR RNA, (A) with* ***3*** *in the absence (○) and presence (●) of an equimolar concentration of MPA, and (B) with the* para *isomer* ***3*** *(●) or the related* ortho *(○) and* meta *(■) isomers. Data are compiled from quantitative analysis of three series of phosphor images such as those shown in Figure 2 and must be considered as a set of averaged values.*

required for the cleavage of TAR RNA by **3**. The metal complex must fit into the cavity created by the trinucleotide bulge to produce RNA-reactive species directed at the opposite U40 reactive site. The specificity of **3** clearly differs from that of a Ni^{II}–Gly-Gly-His metallopeptide[7] and a nonapeptide–cyclen conjugate,[8] both of which cleave the TAR RNA within the C29–G36 apical loop. In contrast, the specificity of our salen–Cu complex is reminiscent of that of the rhodium complex $Rh(phen)_2phi^{3+}$, which induces specific cleavage at residues C39 and U40.[10]

In conclusion, the present study conveys two important pieces of information. First, the combined results of XANES and EXAFS spectroscopy confirm that the hydroxysalen ligand forms a stable complex with Cu^{III} in solution. Second, we show that this unusual Cu^{III} complex functions as a site-specific ribonuclease capable of inducing precise cutting of the TAR RNA at the uridine bulge. These findings open a new avenue to the design of ribonucleases to probe specific structures of RNA.

Experimental Section

The syntheses of the salen–Cu complex **1** and its *para* hydroxy derivative **2** have previously been described[3, 4] and the same procedure was used to obtain the morpholino derivative **3**.

The procedure used for the XANES and EXAFS measurements is given in the Supporting Information.

In vitro transcription of TAR RNA was performed as previously described.[10] The cleavage conditions are described in the Supporting Information.

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Received: August 28, 2002 [Z481]

The Biosynthesis of Indolocarbazoles in a Heterologous *E. coli* Host

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KEYWORDS:

biosynthesis · gene locus · heterologous expression · natural products · rebeccamycin

Rebeccamycin (**3**, Scheme 1), an antitumor antibiotic produced by the bacterium *Saccharothrix aerocolonigenes*, is a prototype of a class of complex natural products called indolocarbazoles. The indolocarbazole alkaloids are an emerging class of natural products that are now typically divided into two major classes according to their structure and mechanism of action.[1] The first class, exemplified by staurosporine and K252a, possess an indolocarbazole subunit wherein the indole nitrogens are bridged by glycosyl linkages; these compounds are potent protein kinase C inhibitors.[2] The second group, exemplified by rebeccamycin,[3] typically carry a single β-glycoside unit, which is

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