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RNA Cleavage by C-1027 Chromophore, an Enediyne Antitumor Antibiotic: High Selectivity to an Anticodon Arm

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Summary: This study demonstrates unique reactivity of the C-1027 chromophore toward a $tRNA^{phe}$. In the presence of Mg^{2+} ions where the $tRNA^{phe}$ attains a stable three-dimensional structure, the enediyne chromophore exhibits high cleavage selectivity to the anticodon arm. The present reactivity of the C-1027 chromophore is useful in development of new chemical probes for mapping of tertiary RNA structure. Considering the paucity of RNA repair mechanisms, RNA may be also an important biological target for certain enediyne antibiotics.

The discovery of enediyne antitumor antibiotics sparks a great deal of excitement in the research area of chemistry, biology, and medicine. C-1027 chromophore is a recently identified member of the potent enediyne antitumor antibiotics.[1] Our laboratory has reported its high ability to cleavage DNA even in the absence of a reductant such as a thiol.[2] This unusual ability is related to the fact that the chromophore contains a labile 9-membered enediyne in contrast to esperamicin, calicheamicin, and dynemicin, agents that contain 10-membered enediyne rings (Scheme 1).[2-5] In this communication, we demonstrate that the C-1027 chromophore interacts with a transfer RNA (tRNA) and produces specific strand breaks in the RNA without a reducing cofactor. This is the first time that RNA has been employed as a substrate for an enediyne antibiotic.

Experimental Section

Materials. As a model RNA substrate, we chose yeast $tRNA^{phe}$, a tRNA whose three-dimensional structure is well known.[6] Yeast $tRNA^{phe}$ was obtained from Boehringer Mannheim. Purified chromoprotein C-1027 was kindly supplied by Dr.T.Otani (Taiho, Tokushima) and nonprotein C-1027 chromophore was extracted from lyophilized C-1027 with distilled methanol at 0 °C. T4 polynucleotide kinase and T4 RNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs, respectively. Distilled water was purified through a Sybron Nanopure II System. All other chemicals used were of commercial reagent grade.

Preparation of tRNA. RNA was 5'-end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP. RNA was also 3'-end labeled by ligation with [5'-^{32}P]$_p$C$_p$, as described by England et al.[7] After end-labeling, $tRNA^{phe}$ was purified by electrophoresis in a 15 %polyacrylamide/7M urea gel.

Cleavage Reactions. A standard reaction mixture (20 μL) contained ^{32}p-end-labeled $tRNA^{phe}$ (3 pmol) and the C-1027 chromophore (10-100 μM) in 10 mM Tris-HCl

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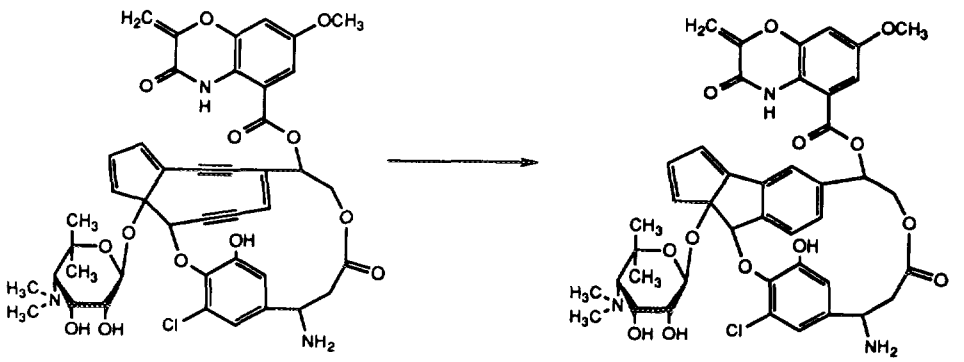

C-1027 chromophore

Scheme 1

buffer (pH 7.4) including 5 mM $MgCl_2$ and 85 mM NaCl. When required, dithiothreitol (1 mM) was added. The cleavage reaction was allowed to proceed for 30 min at 37 °C. Each sample was analyzed on a 15 % denaturing polyacrylamide gel. The cleavage sites were determined by coelectrophoresing with ribonuclease T_1 (G-specific) and alkaline hydrolysis (nonspecific) reactions.

Fluorescence Spectral Measurements. All spectra were recorded on a Hitachi fluorescence spectrophotometer F-3010. The C-1027 chromophore was exited at 320 nm.

Results and Discussion

Typical autoradiographic results with the 5'-end-labeled substrate are shown in Figure 1, and the cleavage data are summarized in Figure 2. In the absence of Mg^{2+} ions where the tertiary structure of $tRNA^{phe}$ is disrupted,[8] the C-1027 chromophore reacted predominantly at stem-loop junctions and bulge loops. It has been reported that grooves of such regions in RNA are accessible to several molecules and also flexible for any structural changes due to the interrupted regularity of the helix.[9-11] We therefore reasoned that the increased accessibility and flexibility of these regions contribute for the selective binding of the bulky chromophore. Once the RNA confines the drug in it, it would sereve as a catalytic template for cycloaromatization of the 9-membered enediyne sector. Indeed, the determination by HPLC method indicated that the chromophore undergoes cycloaromatization at 3.1-fold faster rate in the presence of an equivalent amount of the tRNA than in the absence of the RNA.

In the presence of Mg^{2+} ions where the $tRNA^{phe}$ attains a stable three-dimensional structure,[6,12] by contrast, the enediyne chromophore exhibited higher selectivity. Comparison between parts A and B of Figure 2 reveals that formation of the tertiary structure abolishes most of the cleavage observed in the absence of Mg^{2+}. However, the cleavage at A_{29} and G_{34} remains even in the presence of Mg^{2+} although the cleavage intensity was reduced, and new cleavage bands can be seen at G_{30}, C_{31}, A_{35}, and Y_{37}. All of these six cleavage sites are located in the anticodon arm. These results strongly indicate that magnesium ion-dependent conformational change and stabilization of the

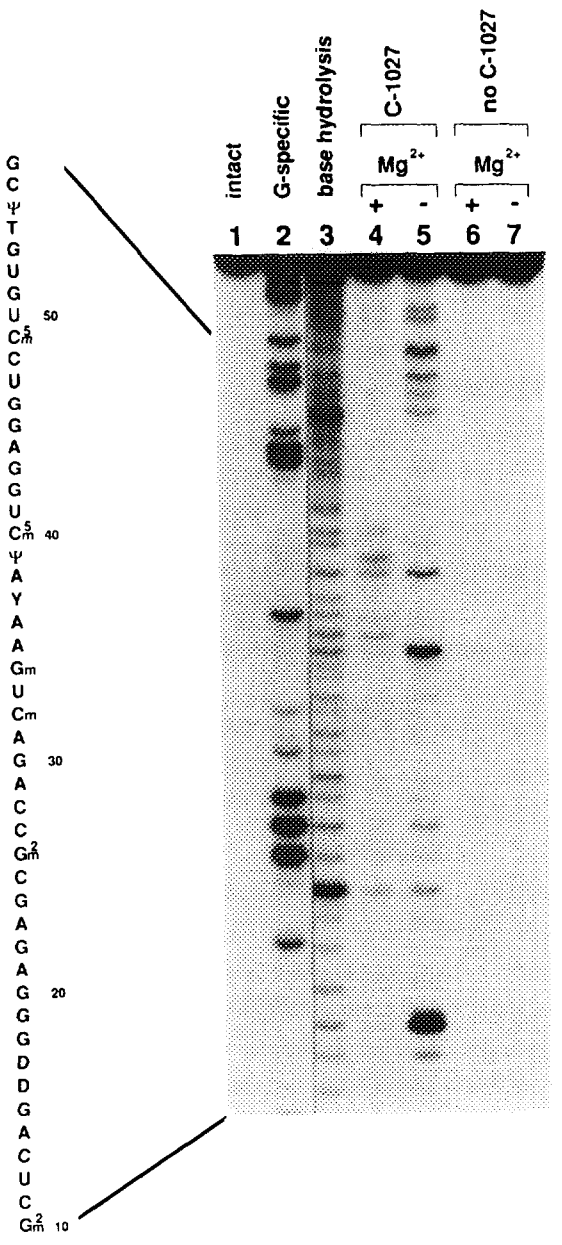

Figure 1. Cleavage of yeast $tRNA^{phe}$ by C-1027. The 5'-end labeled tRNA (11.4 μM nucleotides) was treated with 100 μM C-1027 chromophore at pH7.4 (10mM Tris-HCl) in the presence (lane 4) or absence (lane 5) of 5 mM $MgCl_2$ and 85 mM NaCl. Control reaction mixtures contained no chromophore but received an equal volume of methamol (10% (v/v)) with (lane 6) or without (lane 7) $MgCl_2$ and NaCl. Prior to addition of the chromophore, the samples were heated at 55 °C for 5 min, slowly cooled to room temperature, and then allowed to reaneal at 25 °C for at least 30 min. Each sample was dissolved in 2μL of the loading buffer containing 0.01 % bromophenol blue and xylenecyanol and analyzed on a 15 % sequencing gel. Lane 1 shows intact $tRNA^{phe}$ alone. Lanes 2 and 3 are ribonuclease T_1 digest and partial alkaline hydrolysis ladders, respectively.

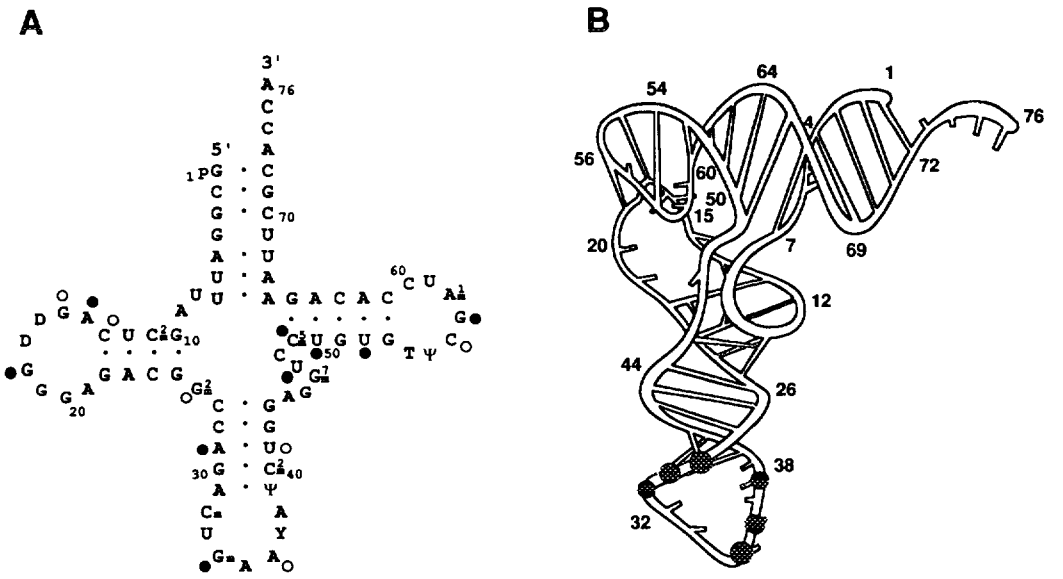

Figure 2. Summary for the cleavage data. Band intensities were quantitated by scanning of the autoradiogram shown in Figure 1 by using a laser densitometer. (A) Cleavage data for C-1027 chromophore in the absence of Mg^{2+} ions mapped onto the secondary structure of $tRNA^{phe}$. The closed and open circles show major and minor sites of cleavage by the chromophore, respectively. (B) Cleavage data in the presence of Mg^{2+} ions mapped onto the tertiary structure of $tRNA^{phe}$. The solid circles indicate the positions of C-1027-promoted strand scission with size corresponding to relative cleavage intensity.

host tRNA have significant effects on the location and efficiency of the RNA cleavage by C-1027. The difference of $tRNA^{phe}$-C-1027 interactions in the absence and presence of Mg^{2+} ions was demonstrated by a fluorescence quenching study (Figure 3). The result reveals that the tRNA binding of C-1027 chromophore is stronger in the presence of Mg^{2+} than in the absence of Mg^{2+}. Magnesium ions may induce local conformational change in the anticodon loop and produce an RNA surface that is accessible to the drug.

When the 3'-end-labeled substrate was treated with this chromophore, the products of the drug reaction migrated far ahead of the markers bearing 5'-hydroxyl groups. Phosphatase (bacterial alkaline phosphatase) treatment quantitatively converted each cleavage band into a band comigrating with the markers, consistent with the formation of fragments having 5'-phosphate termini. On the other hand, the 5'-end-labeled tRNA gave cleavage bands that migrated a little faster than the marker fragments generated by the alkaline hydrolysis. Since the marker fragments are known to have 2',3'-cyclic phosphates at their 3'-termini, a negatively charged species (e.g. glycolate) may be attached to the 3'-terminal phosphoryl group of the breaks produced by the chromophore. Extrapolated from the reaction with DNA,[13] the hydrogen abstraction from the C4' of the ribose may be central to the chemistry of RNA scission by C-1027.

On the other hand, neocarzinostatin chromophore strongly cleaved $tRNA^{phe}$ at 5'-GPu steps such as G_{19}, G_{20}, A_{21}, G_{43}, A_{44}, and A_{66} in the absence of Mg^{2+} ions. When

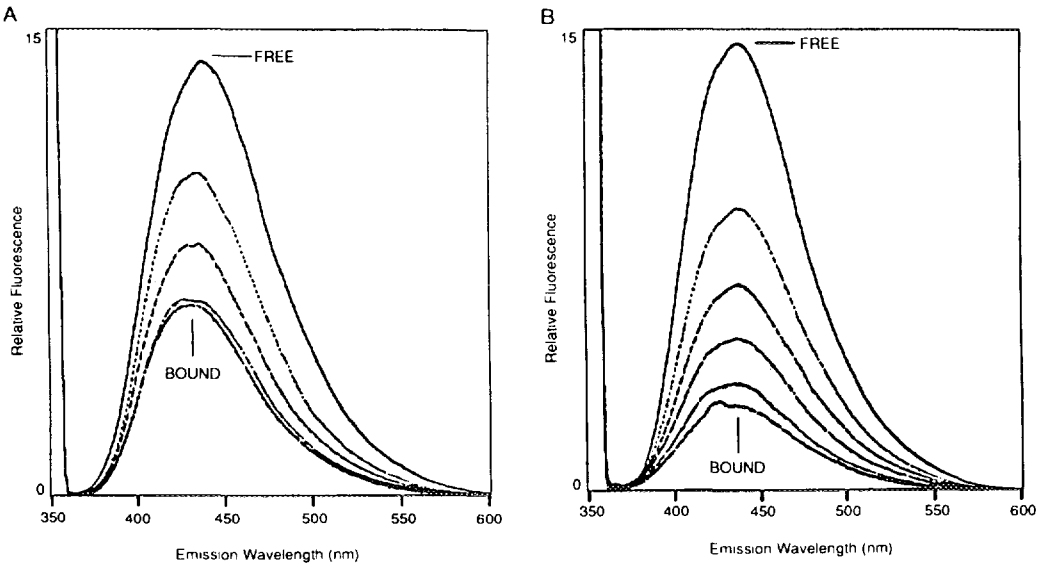

Figure 3. Quenching of the C-1027 chromophore fluorescence by $tRNA^{phe}$ in the absence (A) or presence (B) of Mg^{2+} ions. The RNAs (0-20 μM) were added to a solution of 10 μM C-1027 chromophore at pH 7.4.

the tRNA was treated with this chromophore in the presence of Mg^{2+} ions, however, the cleavage efficiency was considerably reduced in 10 μM Mg^{2+} and completely abolished in 100 μM Mg^{2+} (data not shown). Similar cleavage loss for $tRNA^{phe}$ with tertiary structure has also been observed in studies of bleomycin-iron complex.[14]

How can the C-1027 chromophore achieve the specificity to the anticodon-arm? It would not be correct to assume that these cleavage sites correlate only with exposed backbone surface area of the folded tRNA. In fact, the study on the cleavage of $tRNA^{phe}$ with Fe(II)-EDTA has revealed the exposed backbone areas not only in the anticodon arm but also in many other positions.[15,16] For binding of the C-1027 chromophore, we should consider the structural flexibility of the backbone as well as its solvent accessibility, because some degrees of conformational freedom may be necessary for accommodation of the bulky enediyne chromophore. As evidently shown by crystal structure of $tRNA^{phe}$, there are extensive base-base interactions among the D, TΨC, and variable loops, which stabilize the L-shaped structure of the tRNA and at the same time decrease conformational flexibility of these loops, whereas the anticodon arm is almost free from these interactions (Figure 2B) and then can remain conformationally flexible.[6] Thus, it seems more reasonable to consider that the anticodon-arm specificity is achieved by a combination of the conformational freedom and exposed structure of the anticodon arm. It is also interesting to point out the possibility that helical nature of the anticodon arm may also take part in the specific interaction. In the upper part of the anticodon loop, a Mg^{2+} ion coordinates with a phosphate oxygen and bases via water molecules, accompanied by some local conformational changes.[12] The crystal structure

has revealed that in the presence of Mg^{2+} the anticodon bases stack in single-helical array on the anticodon stem and that the stem has double-helical stacking of the five base pairs.[6] These stacked structures presumably create an RNA groove capable of accomodating the C-1027 chromophore.

In conclusion, this study first demonstrates unique reactivity of enediyne antibiotics toward a tRNA. The present cleavage selectivity may have biological relevance to the action mechanism of C-1027.

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