Flavin-Dependent Photocleavage of RNA at G·U Base Pairs

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When activated by light, photosensitizers can damage cells and organisms by their effects on critical biomolecules. Some of these compounds, such as porphyrins and flavins, are endogenous and are thought to play a role in photocarcinogenesis because their reaction products may cause misreplication of DNA.[1] Flavin derivatives, such as riboflavin or flavin mononucleotide (FMN), can induce photooxidative DNA damage via the generation of an 8-hydroxydeoxyguanosine intermediate.[2−4] In contrast to DNA, little is known about the effect of photosensitizers on RNA. Particularly, it is unknown whether RNA is a target for the photoinduced cleavage by flavins or flavin derivatives.

Here we report that isoalloxazine derivatives, such as lumiflavin (**1**), riboflavin (**2**), and FMN (**3**) (Scheme 1) can cleave RNA molecules with overwhelming specificity at G·U base pairs via a photoinduced mechanism. Recently, we have isolated an RNA aptamer by in vitro selection[5] that specifically recognizes the isoalloxazine moiety of **3** in solution.[6] During the structural characterization of this aptamer,[7] designated as FMN-2 (Figure 1a), we discovered that isoalloxazine derivatives induce strand breakage at the 3′ of the uracil of G·U wobble base pairs in an oxidative photocleavage reaction. This strand breakage is observed with high specificity in other RNA molecules containing G·U pairs embedded within a helix and does *not* require an FMN or isoalloxazine aptamer binding site in the RNA. The specificity of the cleavage reaction is confirmed by testing yeast $tRNA^{fMet}$, yeast $tRNA^{Phe}$, and several synthetic oligonucleotides in isoalloxazine-induced photocleavage.

Incubation of the 5′-^{32}P-end-labeled RNA FMN-2 with 200 μM **3** leads to a 53 nt cleavage product, as shown in Figure 1b. The cleavage occurs downstream of U53, which is part of a G·U wobble base pair within an 11 base pair helical region. The constitution of this stem and the G·U base pair was proven by chemical modification analysis.[7] In the secondary structure, a second G·U wobble base pair is formed by G43 and U6. Cleavage of the 5′-end-labeled RNA at this position could not be resolved in the gel. To test whether cleavage also occurs at the 3′ of U6, we incubated the 3′-end-labeled RNA FMN-2 with **3**. Two cleavage products were obtained, one corresponding to a cut at the 3′ of U53 and another one corresponding to cleavage at the 3′ of U6 (Figure 1c). The strand breakage reaction proceeds in a time dependent manner. Furthermore, cleavage can only be detected upon irradiation with visible light, whereas no product is formed in the dark even at an incubation time of 2 h (Figure 1b). Isoalloxazine derivatives **1**−**3** were all found to promote RNA photocleavage in the same fashion.

At this stage of our experiments, all cleavage reactions had been performed with the RNA aptamer FMN-2 in which the cleavage phenomenon was first observed. The two affected G·U pairs are located outside the well-defined 35mer flavin binding site of the aptamer. However, to exclude that the G·U specific cleavage in this RNA was a function of the specific aptamer binding site for the isoalloxazine ring, we constructed a truncated RNA version of this RNA, designated as FMN-2.del, in which the complete FMN binding site (A67 to G85) was substituted by a GNRA loop and tested it in the cleavage reaction. The RNA FMN-2.del had completely lost its affinity for **1**−**3**; the formation of the 11 base pair stem was confirmed by chemical modification analyses. FMN-2.del was cleaved at exactly the same site as FMN-2. This result clearly shows that the cleavage reaction does not require the FMN recognition site contained in the aptamers. We also confirmed the generality of the cleavage mechanism by using yeast $tRNA^{fMet}$ which contains a single U51·G65 wobble base pair in the T-stem and yeast $tRNA^{Phe}$ which contains a G4·U69 pair in the acceptor stem. A single cleavage site at U51 in $tRNA^{fMet}$ and at U69 in $tRNA^{Phe}$, respectively, was observed, while no other position in these RNAs was affected by the photosensitizer.

In the RNAs described so far, the removed nucleoside downstream of the uracil of the cleaved G·U base pair was either a guanosine or a cytosine. To find out whether cleavage occurs preferentially at G·U pairs with a G or C located at the 3′ of the uracil or whether any base at this position can be removed, we analyzed several synthetic RNA constructs containing one or more G·U pairs with different residues located at the 3′ of

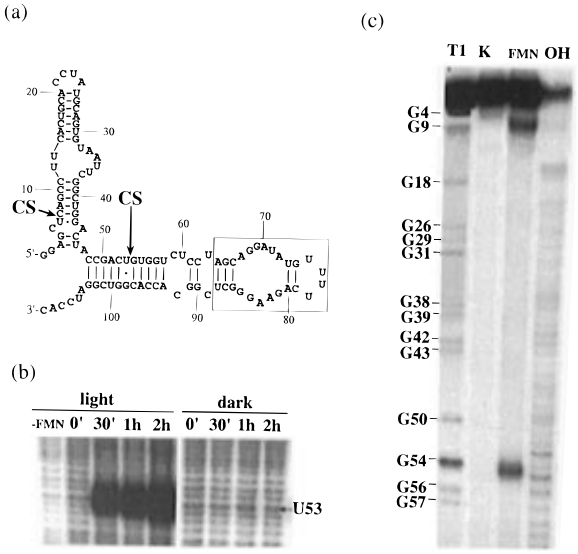
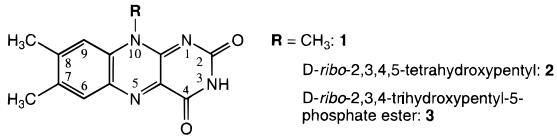


Figure 1. (a) Secondary structure of the FMN-binding RNA aptamer FMN-2, as confirmed by chemical-probing analysis. The isoalloxazine binding site is indicated by the boxed region. The two cleavage sites (CS) at the two G·U wobble pairs present in this RNA are marked. (b) Cleavage of 5′-^{32}P-end-labeled RNA FMN-2 by 200 μM FMN (**3**) in cleavage buffer (250 mM NaCl, 50 mM Tris-HCl, pH 7.6, 12 mM $MgCl_2$) upon irradiation with light at various times and in the dark. (c) Photoinduced cleavage of 3′-^{32}P-end-labeled RNA FMN-2. Abbreviations: T1 RNase T1-sequencing ladder; K, control, incubation for 1 h at 25 °C in cleavage buffer without **3**; FMN, incubation with 200 μM **3** for 1 h at 25 °C in cleavage buffer; OH, alkaline hydrolysis ladder. The cleavage sites correspond to products which terminate at A8 and U55, respectively.

Scheme 1



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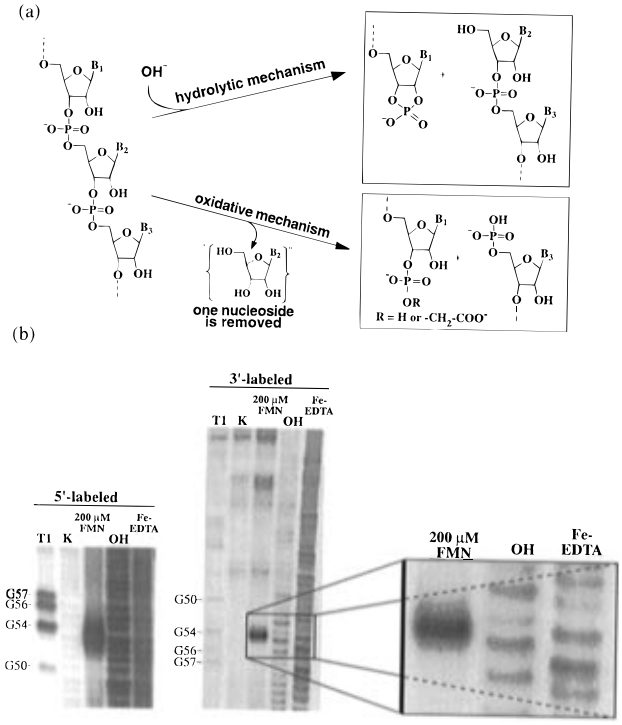

Figure 2. Analysis of the end groups generated during the flavin-induced strand breakage reaction. (a) Mechanisms of hydrolytic and oxidative cleavage reactions of RNA. In hydrolytic pathways, the 2′-hydroxyl group of the nucleotide located upstream of the cleavage site participates in a nucleophilic attack on the phosphorus atom of the cleaved phosphodiester bond resulting in a 2′,3′-cyclic phosphate group and a 5′-hydroxyl group at the ends of the cleaved strands. During oxidative cleavage processes, typically the ribose ring is destroyed which leads to the loss of one nucleotide and the formation of 3′- and 5′-phosphate termini.[12] (b) Cleavage of 5′- and 3′-^{32}P-end-labeled RNA FMN-2 with 200 μM FMN (**3**) and visible light for 1 h at 25 °C. Abbreviations: T1, RNase T1-sequencing ladder; K, control, incubation without **3**; OH, alkaline hydrolysis ladder; Fe·EDTA, Fe·EDTA/H_2O_2 oxidative cleavage.

the uracil. Cleavage occurred independently of the residue located at the 3′ of the uracil involved in the G·U base pairing.

To analyze the end groups formed in the photocleavage reaction, the migration of the product oligonucleotides was compared with those obtained in a hydrolytic and oxidative cleavage reaction.[8] In a hydrolytic mechanism, the 2′-hydroxyl group of the nucleoside which is located at the 5′ to the cleaved phosphodiester bond carries out a nucleophilic attack on the phosphorus atom. This attack results in strand breakage and the formation of 5′-hydroxyl and 2′,3′-cyclic phosphate ends at the cleavage site.[9] In oxidative cleavage processes, the ribose ring is destroyed, leading to the loss of one nucleoside with the generation of a 5′-phosphate on the next residue and a 3′-phosphate or phosphoglycolate terminus on the preceding one (Figure 2a), which migrates differently from the bands resulting from a hydrolytic mechanism.[10,11] The 3′-end-labeled substrate used in Figure 2b permitted analysis of the 5′-termini created at the cleavage site. The cleavage product migrated between the bands of the alkaline hydrolysis ladder but exactly at the same position as the bands generated by Fe·EDTA/H_2O_2 (EDTA = ethylenediaminetetraacetic acid) cleavage, indicating the formation of a 5′-phosphate terminus, which would be in accordance with an oxidative cleavage mechanism.[11,12] The product analysis performed here shows that the 5′-termini generated during the cleavage of RNA by **3** are the same as the ends produced in an oxidative cleavage mechanism.

The chemistry of oxidative cleavage processes also involves the removal of the attacked nucleoside.[10−12] Comparison of the position of the cleavage bands for the 5′- and 3′-labeled substrates relative to the RNase T1-sequencing lanes (Figure 2b) shows that the 5′-labeled product terminates at residue U53, whereas the 3′-labeled product ends at U55. This observation is consistent with the notion that the residue at the 3′ to the uracil of the G·U base pair, G54, is removed in the cleavage process, indicative of an oxidative cleavage mechanism.[10−12]

In contrast to the sharp bands obtained with the 3′-labeled RNAs, we generally observed that the cleavage products derived from the 5′-labeled RNAs were not well resolved in the polyacrylamide gel. Thus, the cleavage reaction appears to oxidatively degrade the sugar in the nucleic acid.[13] In these mechanisms, different 3′-termini are generated.[12]

Interestingly, the cleavage reaction absolutely requires the presence of divalent metal ions, independent of the photosensitizer molecule used. In the presence of alkaline earth metal ions, such as Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} and also Zn^{2+} and Cd^{2+} at concentrations above 1.0 mM, the reaction is able to proceed equally well. No difference in the cleavage intensity was found when reactions contained 2.0 mM EDTA with bivalent metal ions supplemented to the desired effective concentration. Although at present the exact role of the metal ion is unclear, we propose that it might be required first to mediate the recognition of the G·U base pair by the isoalloxazine and, second, might act as a Lewis acid during triplet state activation.

Surprisingly little is known about specificity and even activity of photosensitizer-induced RNA cleavage. The only known example showing that RNAs are affected by photosensitizers is the psoralen-induced photocrosslinking[14] of various positions in ribosomal RNAs, tRNAs, and the spliceosome. The photoreaction of psoralens with RNA, however, occurs via a completely different mechanism than the cleavage mechanism described here.

In summary, these results demonstrate the **1**−**3** promote photoinduced RNA cleavage, with an unexpected selectivity at G·U wobble pairs within RNA helices. The photoreaction results in the removal of one nucleoside downstream of the uracil residue. The effect absolutely requires bivalent metal ions. Our study thus represents the first example in which a structure motif made of an unusual base pair in an RNA is specifically recognized and affected by a low-molecular-weight molecule.

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Supporting Information Available: Experimental details for the photocleavage reaction, table of cleavage results with other RNAs, and data supporting the cleavage with $tRNA^{fMet}$, $tRNA^{Phe}$, and FMN-2.del (5 pages). See any current masthead page for ordering and Internet access information.

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