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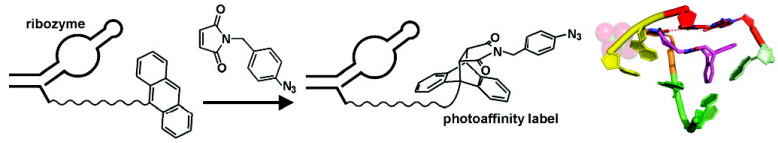

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Probing the Active Site of a Diels−Alderase Ribozyme by Photoaffinity Cross-Linking

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Photoaffinity labeling contributed significantly to our understanding of the structures and mechanisms of protein enzymes.[1] In RNA enzymology, however, there are only very few examples,[2] and this methodology has never been applied to artificial ribozymes that catalyze a diverse set of chemical reactions. Photoaffinity labeling utilizes photoreactive derivatives of compounds that are tightly bound by the enzyme. Upon irradiation, a covalent linkage is generated between enzyme and probe, and the analysis of linkage position(s) allows the identification of residues in the direct vicinity of the bound probe.

Our laboratory has previously reported the discovery of a small RNA capable of catalyzing Diels−Alder reactions.[3] Because of its size and catalytic performance, this ribozyme became subject to many chemical, biochemical, and physical studies, and arguably represents the best-characterized artificial ribozyme known to-date.[4] A milestone in the investigation of this ribozyme was the X-ray crystallographic determination of its three-dimensional structure, both in the unbound form and in complex with the Diels−Alder reaction product made from 9-substituted anthracene and *N*-pentyl maleimide (Figure 1a,b).[5] This crystallographic information allowed a definition of the overall architectural principles and identification of the residues that constitute the catalytic pocket. Recent bulk and single-molecule experiments in solution, however, revealed the existence of different folding states, but knowledge about the catalytic relevance and molecular properties of these conformers is lacking.[4f] In this study, we use photoaffinity cross-linking to probe structural features of Diels−Alderase active site conformations in solution.

The compound currently known to have the highest affinity to the active site of the Diels−Alderase ribozyme is one enantiomer of the Diels−Alder reaction product.[4b] Thus, a photoaffinity probe was designed based on this scaffold, containing a *p*-azidobenzyl chain attached to the imide ring as the photoreactive group. Furthermore, a hexa(ethylene glycol) tether was incorporated between the catalyst's 5′-phosphate and the probe's bridgehead position to ensure a 1:1 stoichiometry and to increase the local concentration of affinity label in the vicinity of the active site (Figure 1c).

Retrosynthetic analysis revealed that such a compound can be synthesized easiest by using the ribozyme itself: In the ribozyme-catalyzed reaction, the desired product enantiomer is formed almost exclusively, and both benzylic maleimides and covalent tethering of the anthracene diene had been shown previously to be compatible with the function of this ribozyme.[3,4b] Moreover, the ribozyme is known to be highly active in a bipartite version, allowing the assembly of the catalyst from an unmodified 38-mer strand and an anthracene-tethered 11-mer (Figure 1c) and thereby facilitating the analysis of the cross-links, as interstrand cross-links would cause large changes in molecular size.

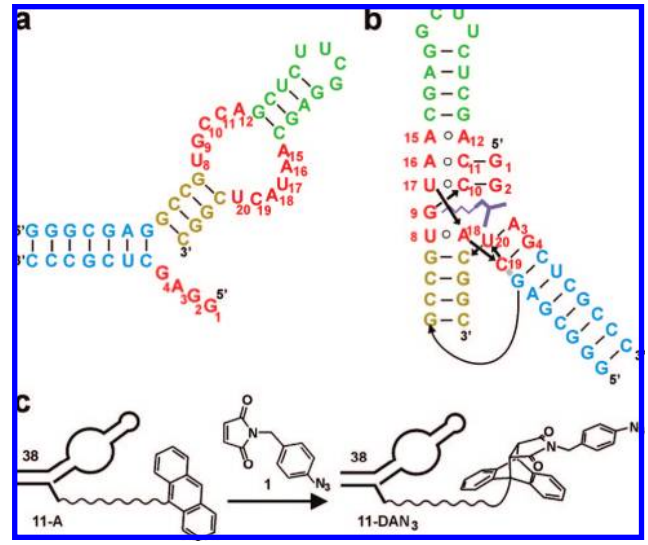

Figure 1. Secondary (a) and tertiary (b) folds of the Diels−Alder ribozyme. Numbering as in ref 3; (c) ribozyme-catalyzed synthesis of a photoaffinity label.

p-Azidobenzylmaleimide **1** is easily accessible in six steps starting from *p*-toluidine.[6] Irradiation of ethanolic solutions of **1** at different wavelengths yielded characteristic changes in the UV spectra, thereby confirming the formation of reactive keteneimine species.[7] As expected, the maleimide was accepted as a substrate by the Diels−Alderase ribozyme.

For the preparative synthesis of target compound 11-mer-DAN$_3$, 38-mer ribozyme strand and 11-mer-tether-anthracene strand (11-mer-A) were mixed in buffered solution, **1** was added, and the reaction was allowed to proceed for 10 min (Figure 1c), generating the desired product in typical yields of ∼65% with ee values >90% (*S*,*S*-enantiomer).[4d] Radioactive ^{32}P labels were introduced either pre- or postsynthetically, and product 11-mer-DAN$_3$ was either isolated and purified, or used directly for in situ photoaffinity cross-linking. Figure 2a shows the gel-electrophoretic analysis of photocross-linking. Unlabeled, HPLC-purified 11-mer-DAN$_3$ was mixed with 5′-^{32}P-labeled 38-mer under Diels−Alderase standard reaction conditions (buffer pH 7.4, 300 mM NaCl, 80 mM $MgCl_2$) and subjected to irradiation at 302 nm for 20 min. Two new slower moving bands were observed. Control experiments using only the 38-mer, or substituting the 11-mer-DAN$_3$ by 11-mer-A or 11-mer-DABM (a Diels−Alder product originating from benzylmaleimide that lacks the azido group) yielded no cross-link bands.

As the formation of the catalytically competent structure depends on divalent metal ions,[3,4f] cross-linking was studied as a function of the Mg^{2+} ion concentration (Figure 2b). At 0 mM Mg^{2+} we observed a weak band with the same mobility as the one seen earlier

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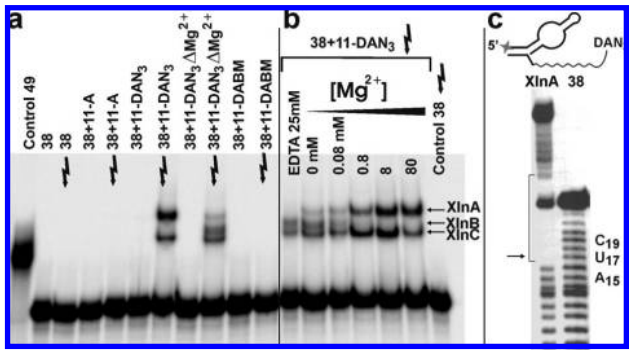

Figure 2. (a) Cross-linking of the bipartite ribozyme. Denaturing PAGE autoradiogram of irradiated (indicated by flash) and nonirradiated samples containing 5′-^{32}P-labeled 38mer and different unlabeled 11-mer samples. (b) Mg^{2+} ion dependence of cross-linking. Bands corresponding to cross-linked species are indicated as XlnA, XlnB, and XlnC. (c) Mapping the photoaffinity cross-link XlnA by limited alkaline hydrolysis.

(XlnC), and a rather diffuse band (XlnB) that disappears completely with increasing Mg^{2+} concentration. The lower band (XlnC) shows an intensity maximum at 8 mM Mg^{2+}. The intensity of the upper band (XlnA) increases continuously with the Mg^{2+} concentration with a maximal cross-link yield at 80 mM where catalytic activity is highest.

To determine the number of different species and the cross-link position(s), the respective bands were excised from the gel, eluted, ^{32}P-labeled at different positions, subjected to limited alkaline hydrolysis, and reanalyzed by PAGE. Band XlnA derived from the 5′-^{32}P-radiolabeled 38-mer and unlabeled 11-mer-DAN_3 displayed a large gap in the hydrolysis ladder starting at nucleotide U17 (Figure 2c). While for all nucleotides from nucleotide A16 down to the 5′-end the hydrolysis bands comigrated with those of the unmodified 38-mer, all bands from nucleotide U17 to the 3′-end were retarded, strongly suggesting the attachment of the photoaffinity probe to position U17 in the 38-mer strand. At the cross-link site, a tribranched structure is formed, resulting in RNA species with lower electrophoretic mobility. Quantitative analysis of the band densities by phosphorimaging indicates that U17 is the only significant cross-link position inside this population. The cross-link between the azidobenzyl group and U17 was further substantiated by variations of the procedure, using either 3′-labeled 38-mer or 3′-labeled 11-mer-DAN_3. Applying the same methodology, the lower cross-link band XlnC could be assigned to nucleotide C10, whereas such an assignment failed for the diffuse band XlnB (Figure 2b).

Thus, using photoaffinity probe 11-mer-DAN_3, three different cross-links could be identified in this study, depending on the conditions used: In the absence of divalent metal ions, cross-linking is apparently unspecific. At intermediate metal ion concentrations, nucleotide C10 is the predominant cross-linking site, while at high concentration nucleotide U17 was preferentially cross-linked. This metal-ion dependence indicates the existence of two different RNA folds with affinity to the Diels−Alder product. Remarkably, the metal ion dependent intensity changes of cross-link bands A, B, and C parallel the changes in the proportions of folded, unfolded, and intermediate populations, respectively, observed by single-molecule FRET.[4f] This may suggest that the partially folded intermediate FRET conformation is able to bind the Diels−Alder product, which would explain why the ribozyme is—despite frequent breakdown of the folded state[4f]—strongly product-inhibited.[4b]

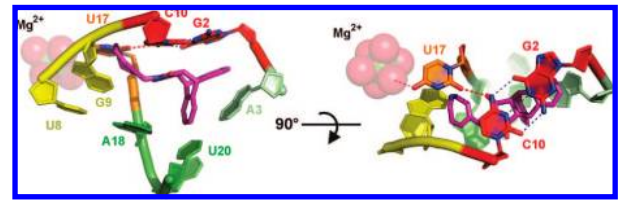

Figure 3. Active site of the Diels−Alderase ribozyme from the crystal structure of the ribozyme-product complex[5] with a proposed conformer of the photoreactive product which is shown in the activated keteneimine form, calculated using MOPAC: front view (left) and a 90° turned top view (right).

The two nucleotides U17 and C10 identified here are known to be constituents of the active site and to make direct contacts with the maleimide portion of the Diels−Alder product (Figure 3).[5]

In conclusion, we located the active site of the Diels−Alderase ribozyme in solution by photoaffinity cross-linking. We identified two specific nucleotides, C10 and U17, that are cross-linked by an azide-substituted product analogue, and which of these two nucleotides is predominantly cross-linked depends on the Mg^{2+} ion concentration. This metal-ion dependence reveals conformation-dependent interactions between ribozyme and probe. While the identified cross-link positions are in excellent agreement with the available X-ray crystal structure, the design of photoaffinity probes does not require such structural information. The results suggest that for other ribozymes, a similar cross-linking approach could be very informative even without a crystal structure available, which is the situation for most other artificial ribozymes. The present work demonstrates the utility of photoaffinity cross-linking as an empirical approach that is applied here for the first time to an artificial ribozyme. The ribozyme itself proved to be a useful catalyst for the synthesis of the photoaffinity probe.

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Supporting Information Available: Full experimental procedures and analytical characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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