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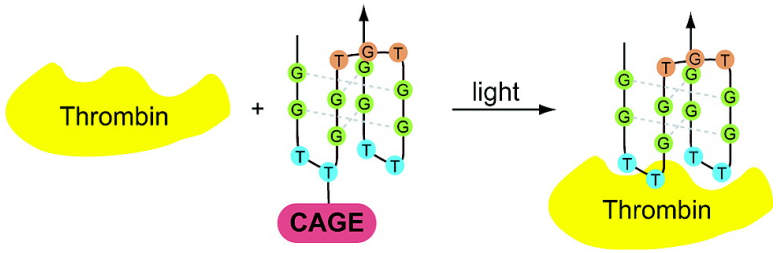
J. Am. Chem. Soc., **2005**, 127 (3), 822-823• DOI: 10.1021/ja043285e • Publication Date (Web): 24 December 2004



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Published on Web 12/24/2004

Light Regulation of Aptamer Activity: An Anti-Thrombin Aptamer with Caged Thymidine Nucleobases

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Aptamers are short single-stranded nucleic acids that fold into well-defined three-dimensional shapes. They bind with high affinity and specificity to their respective target molecules and possess high inhibitory potential. These characteristics make them very interesting tools for molecular biology as well as for diagnostic and therapeutic applications.[1]

One of the most intensively studied aptamers is a 15mer ssDNA molecule (**A**$_1$, Figure 1) that binds to and inactivates α-thrombin, which is a key player in the blood clotting cascade. The aptamer folds into a stable G-quartet structure and only consists of six thymidine and nine guanosine nucleotides.[2]

The control of inhibitory function is a challenging issue, and for example a recent study reported the use of antisense oligonucleotides to inhibit aptamer function, interfering with the blood clotting cascade.[3] However, we try to gain control over the exact temporal and spatial availability of the aptamer's inhibitory function using light as a highly orthogonal trigger signal. If photolabile groups can be introduced in positions which are important for the aptamer's function, the aptamer can be temporarily inactivated and the full function can be recovered by controlled irradiation with light. This strategy is commonly referred to as "caging",[4] but only few studies exist in which nucleic acids are caged: In some of them the backbone phosphate groups of plasmids and mRNA have been caged statistically.[5] Other groups have caged the 2′-positions in ribozymes.[6] However, since we see the nucleobases as the key players and since we want to rationally choose the position of the caged residue and incorporate as few modifications as possible, we have chosen to prepare and study caged nucleobases. In a previous study we have established the synthesis of caged thymidine phosphoramidites and demonstrated their use to control T7 RNA polymerase-mediated transcription by light.[7] Recently a study by another group has been published in which a caged adenosine was used to trigger the activity of a DNAzyme with light.[8] Here we would like to report on the adaptation of our strategy of caged thymidine nucleobases to aptamer technology.

On the basis of the crystal structure[9] (PDB ID 1HAO) of thrombin with the 15mer ssDNA aptamer **A**$_1$, we developed and synthesized aptamer variants (**A**$_2$−**A**$_5$) that carry the caged thymidine residue T^{NPP} in various positions (Figure 1). We assumed that caged residues in position T4 (as in the caged aptamer **A**$_2$), T13 (as in **A**$_3$), or both thymidine positions (as in **A**$_4$) would inhibit the interaction of the aptamer with thrombin and caging groups in position T9 (as in **A**$_5$) which is not involved in binding thrombin would presumably not change the aptamer's activity. As a positive control we also included a mutant of the aptamer (**A**$_6$) that has an adenosine in position T4 instead of a thymidine residue and does not bind to thrombin. To test our design strategy and to determine the dissociation constants (K_D) of the caged aptamers and their light-activated counterparts, we performed interaction studies with radioactively labeled aptamers.

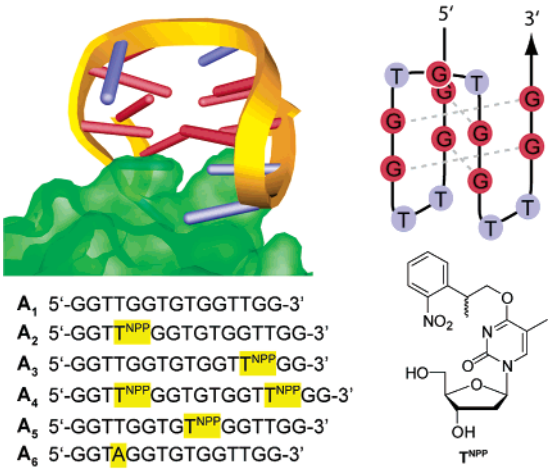

Figure 1. (Top) Schematic representation of a crystal structure of the ssDNA aptamer **A**$_1$ with its protein target thrombin.[9] G and T residues are shown in red and blue, respectively. The van der Waals surface of the protein is shown in green. (Bottom) Sequences of the (caged) aptamers **A**$_1$−**A**$_6$ synthesized for this study.

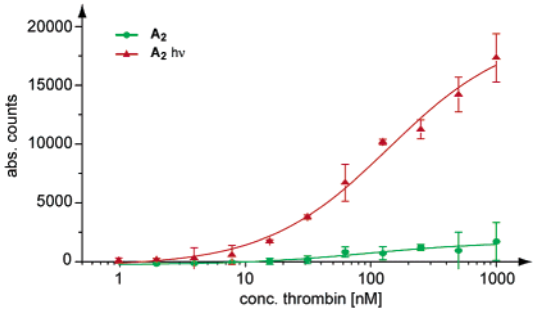

Figure 2. Results of a filterbinding analysis with thrombin and the aptamer **A**$_2$ with and without irradiation.

Table 1. Dissociation Constants (K_D) of the Respective Aptamers and Thrombin in nM

aptamer	without irradiation	after irradiation
A$_1$	99 ± 21	
A$_2$	not detectable	139 ± 38
A$_3$	not detectable	121 ± 27
A$_4$	not detectable	122 ± 31
A$_5$	64 ± 10	101 ± 14
A$_6$	not detectable	

As shown in Table 1 and Figure 2 no interaction of the caged aptamers with thrombin was detected. However, irradiation with light essentially restored the thrombin binding activity. The resulting K_D values of the light-activated aptamers **A**$_2$−**A**$_5$ are comparable to the one of the wild-type aptamers **A**$_1$ within error limits, whereas the mutant **A**$_6$ does not show significant binding to thrombin. In

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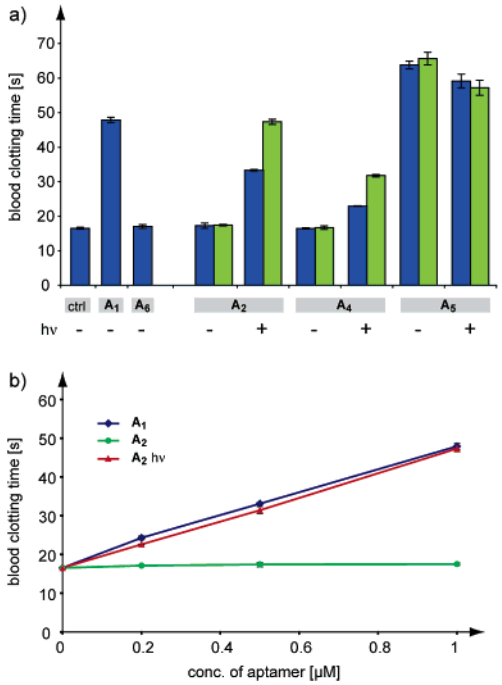

Figure 3. Blood clotting times measured in the presence and absence of caged and light-activated aptamers. Light irradiation was performed in phosphate buffer at pH 7.4 (blue columns) or pH 11.0 (green columns).

accordance with the crystal structure and our design strategy the aptamer $\mathbf{A_5}$ with the photolabile group in position T9 (which is not involved in binding) still interacts with thrombin, in both its caged and uncaged state. It even turned out that $\mathbf{A_5}$ is a slightly better inhibitor which might be due to conformational changes.

Next, we analyzed the effect of the caged and light-activated aptamers on the thrombin-dependent blood clotting time. Therefore, the increase of the blood clotting time in human plasma was measured, after addition of a 1 μM concentration of wild-type aptamer $\mathbf{A_1}$, the mutant $\mathbf{A_6}$, and the caged aptamers $\mathbf{A_2}$, $\mathbf{A_4}$, and $\mathbf{A_5}$, both before and after light activation (Figure 3).

The wild-type aptamer $\mathbf{A_1}$ causes an increase of the blood clotting time by a factor of 3 compared to the control reaction without oligonucleotide or with the mutant aptamer $\mathbf{A_6}$, which has also been found previously in the literature.[2] In accordance with our design and as expected from the affinity measurements, the aptamer $\mathbf{A_5}$ with the photolabile group in the position which is not involved in binding could still inhibit thrombin. The singly and doubly caged aptamers $\mathbf{A_2}$ and $\mathbf{A_4}$ with the photolabile groups in the right position, however, were completely inactive. This clearly demonstrates that already one modified nucleotide in a key location can be enough to completely mask an aptamer's function.

As shown in Figure 3a the full activity expected from a completely uncaged aptamer was not obtained after irradiation with light (366 nm) in a phosphate buffer at pH 7.4 ($\mathbf{A_2}$ and $\mathbf{A_4}$, blue bars in Figure 3a) but could be restored by irradiation in phosphate buffer at pH 11.0 (green bars in Figure 3a). Complete rescue of the activity was even more difficult in the case of the doubly caged aptamer $\mathbf{A_4}$. While it is of course desirable to fully restore the activity, it is not uncommon that irradiation of caged compounds does not result in a full recovery of their activity.[5b] In HPLC studies we found out that in the case of our structured aptamers a byproduct was formed that did not react any further upon irradiation. This is in contrast to our previous results in which we clearly showed that the NPP group is superior to other commonly used photolabile groups, especially considering the yield of the deprotection reaction. Quantitative formation of the uncaged product was observed even when three T^{NPP} residues were present in an oligomer.[7] Thus, it appears that the deprotection characteristic is sequence dependent. In contrast to the oligonucleotides in our previous study, the aptamer $\mathbf{A_1}$ is highly structured and has a high G content. It has been shown in a mechanistic model study that the pH can be important in the photodeprotection of the NPP group.[10] The fact that photodeprotection can be difficult underlines our efforts to include as few caging groups in rationally chosen key positions as possible.

In summary, we have provided further evidence that the strategy of caging nucleobases is a general one and can be expanded to more nucleotide-based applications, like aptamers in this case. As in the preceding study[7] one residue can be chosen and modified, on the basis of rational design, which results in complete masking of the function. Unlike in our previous study the photodeprotection was not quantitative under physiological pH conditions. This calls for a systematic study of different caging groups in the future. Light-induced aptamers will provide an opportunity for the spatial and temporal control of bioactive molecules and will enhance the application of aptamers in the context of cellular and biological environments.

Acknowledgment. The authors thank Prof. B. Pötzsch (University Hospital Bonn) for allowing them to do the blood clotting measurements in his facilities, as well as W. Lenz for his skillful technical assistance and Dr. M. Engeser for measuring the MS spectra. Prof. M. Famulok is gratefully acknowledged for his kind support. This work was funded by a Liebig-Fellowship of the "Verband der chemischen Industrie" (Germany) for A.H.

Supporting Information Available: Experimental details on DNA synthesis, interaction analysis, blood clotting assays, and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA043285E