Satellite Hole Spectroscopy of Thrombin – Aptamer Complexes

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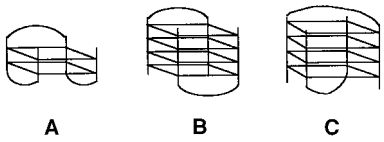
KEYWORDS:

aptamers · dyes · molecular recognition · protein – DNA interactions · satellite hole spectroscopy

Thrombin is a clotting enzyme that plays a central role in both thrombosis and hemostasis[1] and hence is a major target in anticoagulation and vascular disease therapy.[2] Recently, a 15-mer aptamer (Apt) oligonucleotide, d($G_2T_2G_2TGTG_2T_2G_2$), has been shown to exhibit a high thrombin-binding affinity and to inhibit thrombin-induced fibrin clot formation.[3] Structural studies using NMR spectroscopy[4] and X-ray crystallography[5] have demonstrated that Apt adopts a very stable unimolecular quadruplex structure, consisting of two guanine quartets connected by one TGT loop folded over one G quartet and two TT loops folded under the other quartet (→**A**). The G-quartet structure formed by Hoogsteen hydrogen bonds between the four guanine bases has also been observed in telomere DNA.[6] NMR-spectroscopic[7] and X-ray studies[8] on d($G_4T_4G_4$) from the telomere DNA of *Oxytricha* (this sequence is abbreviated as Oxy) have shown that two hairpin duplexes can dimerize into different structural isomers of the quadruplex (→**B** and **C**). The similar quadruplex structures of Apt and Oxy and the important role of thrombin in blood coagulation stimulated us to compare the structures of these thrombin – oligonucleotide complexes and to develop a new approach for detecting thrombin – DNA recognition.



Recently, we have compared the nonresonant holes (NRHs) in the satellite hole (SH) spectra of 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid succinimidyl ester (BODIPY SE) and of the BODIPY moiety covalently attached to various oligonucleotides doped in glycerol/water (Gl/H_2O) glass.[9] When laser excitation is tuned to wavelengths in the region of vibronic transitions in the inhomogeneously broadened absorption band, a number of NRHs resulting from Franck – Condon (FC) active vibrational modes can be produced in the SH spectrum.[10] Note that each NRH can be viewed as an individual electronic transition of its own vibronic transition. As a result, the intensity of NRH can be enhanced by the inverse FC factor.[11] Furthermore, thermal broadening of the linewidth is significantly reduced at $T \approx 6$ K. Our SH spectroscopic results showed that distinct NRHs are very useful to probe the binding modes of BODIPY to the oligonucleotides and their conformations. An example for the chemical structure of BODIPY covalently attached through a hexylamine spacer to the 5′-end of the oligonucleotide d(G_{10}), BODIPY – d(G_{10}), is shown in Figure 1.

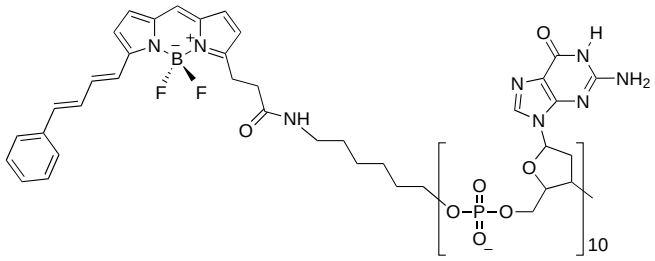


Figure 1. *Schematic representation of the covalent adduct BODIPY – spacer – d(G_{10}).*

In this work, we first examine whether the SH spectroscopic method can be used to probe thrombin – Apt recognition. The absorption and SH spectra of free BODIPY SE in the presence of thrombin, the BODIPY – Apt adduct without thrombin, and BODIPY – Apt in the presence of thrombin are compared. In addition, we ascertain whether or not the G-quartet structure of other DNA sequences can be recognized by thrombin and whether or not these structures are distinguishable. Toward this end, we have compared the SH spectra of Oxy as well as the hairpin duplex d($CGCGT_4CGCG$) (abbreviated as HD) in the presence of thrombin. The HD sequence cannot form a G-quartet structure.[12]

Figures 2 a – c show the HPLC analyses of BODIPY – HD, BODIPY – Apt, and BODIPY – Oxy (detection at 260 and 590 nm). The chromatograms revealed two major peaks, one at ca. 26 min retention time (fraction A) and another, predominant one at ca. 41 min retention time (fraction B). In our previous studies,[9] the SH spectrum of BODIPY – HD-A suggested that the thymine residues in the G-rich sequences can interact with the BODIPY chromophore to form an intramolecular loop. Since the SH spectrum of BODIPY – HD-B is similar to the spectrum of free BODIPY SE, it is surmised that the BODIPY moiety is located outside the hairpin duplex.[9] With Apt and Oxy, we find that much more B adducts are produced than A adducts. The hairpin structures of Apt and Oxy can subsequently rearrange to yield a G-quartet structure. Both NMR spectroscopic and X-ray crystallographic results have indicated that Apt adopts a G-quartet structure. Therefore, we anticipate that both BODIPY – Apt-B and BODIPY – Oxy-B adducts exist predominantly in the G-quartet

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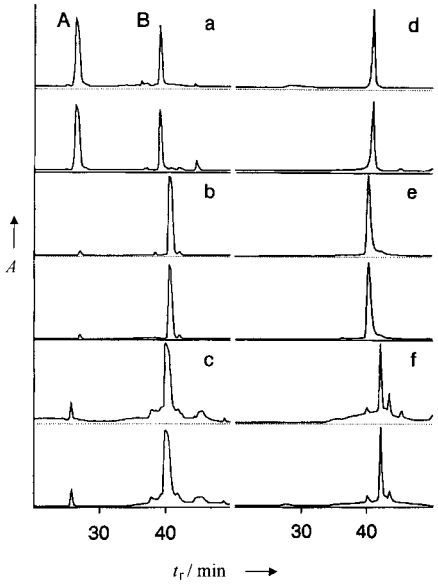

Figure 2. *HPLC analyses of BODIPY – HD (a), BODIPY – Apt (b), and BODIPY – Oxy (c) taken at absorbances of 260 nm (upper trace) and 590 nm (lower trace). Figures d – f show the chromatograms of the collected B fractions from the HPLC runs in figures a – c.*

form. Figures 2 d – f show no signs of conversion from the B adduct to the A adduct, implying that the B adduct is stable.

Figures 3 a – c show the preburn and postburn spectra of BODIPY SE/thrombin, BODIPY – Apt, and BODIPY – Apt/thrombin doped in Gl/H_2O glass taken at 6 K, respectively. The absorption band is located at ca. 596 nm for BODIPY SE/thrombin, ca. 600 nm for BODIPY – Apt, and ca. 606 nm for BODIPY – Apt/thrombin. The red-shift of the absorption band provides evidence for the interaction between BODIPY – Apt and thrombin. Figures 3 d – f show the SH spectra obtained from the difference between each preburn and postburn spectrum recorded at $\lambda_B \approx 585$ nm. The SH spectra show a blue-shift from

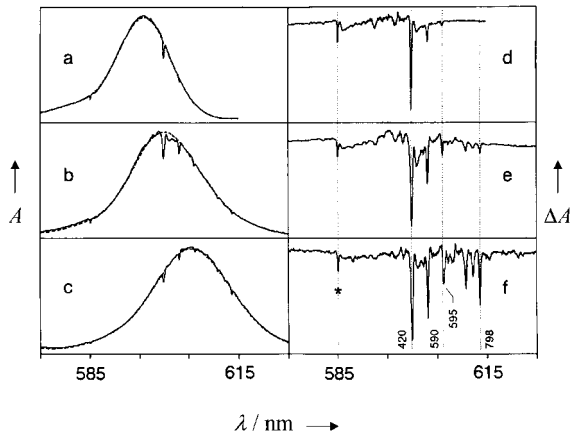

Figure 3. *Preburn and postburn spectra of BODIPY SE/thrombin (a), BODIPY – Apt (b), and BODIPY – Apt/thrombin (c) doped in Gl/H_2O glass recorded at $\lambda_B \approx$ 585 nm and the corresponding satellite hole spectra (d – f). The pulse energy was ca. 1.5 μJ with a burn time of 10 min for each spectrum. * denotes the burning position. Prominent nonresonant holes are labeled with excited-state vibrational frequencies.*

the 590 cm^{-1} band in the SH spectra of BODIPY SE/thrombin and BODIPY – Apt to 595 cm^{-1} in the SH spectrum of BODIPY – Apt/thrombin. Considering the red-shift of the absorption band, Figures 4 a – c show the SH spectra recorded at $\lambda_B \approx 575$ nm for BODIPY SE/thrombin, $\lambda_B \approx 580$ nm for BODIPY – Apt, and $\lambda_B \approx$ 580 nm for BODIPY – Apt/thrombin.

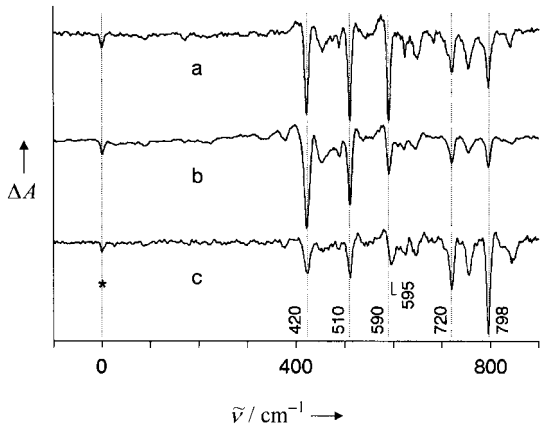

Figure 4. *Satellite hole spectra of BODIPY SE/thrombin (a), BODIPY – Apt (b), and BODIPY – Apt/thrombin (c) doped in Gl/H_2O glass recorded at $\lambda_B \approx$ 575, 580, and 580 nm, respectively.*

Figure 5 shows the absorption and SH spectra of BODIPY – Oxy and BODIPY – HD in the presence of thrombin doped in Gl/H_2O glass recorded at $\lambda_B \approx 585$ nm. The absorption maximum is shifted from ca. 600 nm to ca. 602 nm, and the NRH at 590 cm^{-1} is shifted to 595 cm^{-1} in the SH spectrum of BODIPY – Oxy upon

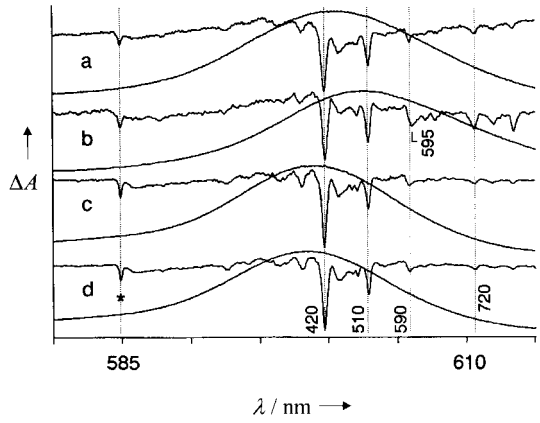

Figure 5. *Absorption and satellite hole spectra of BODIPY – Oxy (a), BODIPY – Oxy/thrombin (b), BODIPY – HD (c), and BODIPY – HD/thrombin (d) doped in Gl/H_2O glass recorded at $\lambda_B \approx$ 585 nm.*

mixing with thrombin. However, no appreciable frequency shifts in the SH and absorption spectra are observed for BODIPY – HD in the presence of thrombin. A similar frequency shift is observed for SH in BODIPY – Apt and BODIPY – Oxy, but not in BODIPY – HD, suggesting that the G-rich structure is a major factor for the recognition of Apt by thrombin.

Molecular modeling and chemical modification studies suggested that the anion-binding exo-site and the putative heparin recognition site are the two most likely sites for the binding of thrombin to Apt.[13] Although two different structures of thrombin – Apt complexes have been proposed on the basis of NMR-spectroscopic[14] and X-ray-crystallographic data[5], both studies have suggested that Apt binds to the surface of thrombin to prevent fibrin clot formation. In addition, NMR-spectroscopic studies[13c] have suggested that the G-quartet structure of Apt is not changed upon binding to thrombin. Since BODIPY is a hydrophobic molecule, the hydrophobic effect can steer BODIPY toward thrombin. As a result, BODIPY covalently attached to Apt can interact with the surface of thrombin when thrombin is bound to Apt.

In our earlier work,[9] we found that the BODIPY moiety is a very sensitive probe for the study of dye – DNA interactions. The most sensitive site involves the BF_2 moiety. Mode assignment suggested that the NRH at 590 cm^{-1} is mainly determined by the strong BF_2 scissoring. In the present study, the frequency shift from the 590- to the 595-cm^{-1} band clearly indicates that the BF_2 scissoring is perturbed by thrombin. On the other hand, the absence of appreciable differences for other prominent NRHs supports the assumption that the BF_2 group of BODIPY couples with the surface of thrombin. Furthermore, it is important to note that all SH spectra were reproduced even when the samples were cycled between room temperature and 4.2 K for many times. We believe that the interaction of BODIPY with thrombin resulting from the binding of Apt to thrombin also occurs at room temperature.

The spectral results obtained for BODIPY-modified Apt, Oxy, and HD allow us to delineate some of the factors important for thrombin recognition. Our data show that thrombin can recognize Apt and Oxy, but not HD. Since formation of a hairpin duplex structure is possible for all three complexes, recognition cannot originate from the presence of a hairpin duplex structure alone. Although we do not know how Oxy inhibits thrombin at the molecular level, our results also support the notion that the G-quartet structure formed by Hoogsteen-type base pairing is closely related to the recognition of thrombin by Apt. Understanding the structural motifs that are important for the interaction and the sequence specificity of the DNA binding to the protein is a central theme in the regulation of a number of biological processes and for pharmacological applications.

We have shown here that SH spectroscopy of a sensitive dye, covalently attached to a specific DNA sequence, can be a useful tool for the detection of certain protein – DNA recognition events. It is conceivable that this approach could be developed further for the diagnosis of a number of genetic diseases. Drugs are normally designed on the basis of how they interact with specific targets to modify their biological function in such a way that they benefit the host system. Our results suggest that the covalent attachment of a dye molecule to different DNA oligonucleotides need not break the binding between thrombin and these aptameric sequences. It also implies that a drug can be covalently attached to a specific DNA sequence and reach its target as a result of protein – DNA binding.

Experimental Section

The experimental setup for the SH spectroscopy was as described previously.[15] Holes were produced by using a sync-pumped dye laser pumped by a mode-locked and Q-switched Nd:YAG laser. The dye laser emitted trains of ca. 10 pulses separated by 13 ns at a repetition rate of 500 Hz. The full width at half maximum (FWHM) of the dye laser was ca. 2 cm^{-1}. The absorption spectrum was obtained by dispersing the output of a xenon arc lamp (Oriel 6083) through a double-beam spectrometer with a resolution of ca. 0.03 nm. The spot size of the burning beam was ca. 1 mm and that of the reading beam was ca. 200 μm.

Human plasma thrombin was purchased from Calbiochem-Novabiochem Corporation and BODIPY SE was purchased from Molecular Probes (catalog no. D-2228). They were used without further purification. The 1:1 adducts of BODIPY covalently attached to oligonucleotides were prepared as described previously.[9] An HPLC apparatus (Shimadzu, SCL-6A) with a diode array detector (SPD-M10A) was employed to isolate the dye-labeled oligonucleotide. Eluents collected at different retention times were examined by their absorbance at 260 nm for the oligonucleotide and at 590 nm for the dye moiety, respectively. The dye-labeled oligonucleotides were dried and redissolved in a mixture of glycerol and water (5:4, v/v). The concentration of BODIPY-modified oligonucleotide and thrombin after mixing is approximately the same. Solutions were injected into a quartz tube with an inner diameter of ca. 1.5 mm and placed in a brass holder with several drilled holes of ca. 1 mm in diameter. Clear glasses were normally formed from room temperature to 6 K by introducing the samples directly into a Janis dewar.

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Synthesis of Characteristic H-Ras Lipopeptides by Employing Noble-Metal-, Acid-, and Reduction-Labile Blocking Groups

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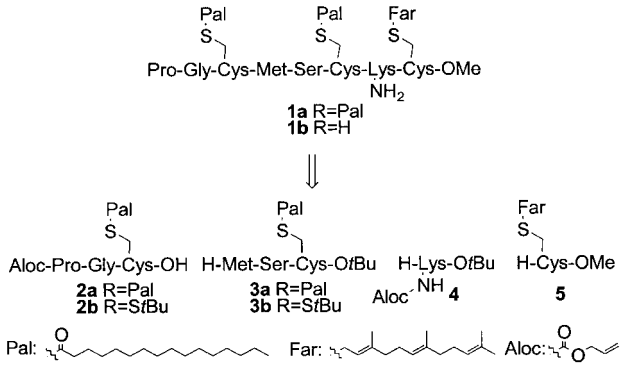
peptides · protecting groups · protein lipidation · Ras proteins · signal transduction

Numerous proteins covalently modified by fatty acid thioesters or prenyl thioethers are involved in biological events of paramount importance, such as signal transduction, organization of the cytoskeleton, and vesicular transport.[1, 2] Lipidation of these proteins is a prerequisite to correct biological function, and the lipid groups are believed to participate in protein – protein and protein – membrane interactions which, for instance, may determine the selective intracellular localization of lipid-modified proteins.[2–5] By combining techniques of organic synthesis and molecular biology, we have recently developed a general biological readout system that allows to determine and quantify the ability of a given lipidated peptide moiety to direct a given protein to the plasma membrane.[6] The key step of this method consists of the coupling of a mutant Ras protein to different selectively functionalized lipidated peptides. The scope of this system critically depends on the availability of efficient methods for the synthesis of multifunctional and sensitive farnesylated and palmitoylated peptides, even in selectively deprotected form, if so required. Farnesylated and palmitoylated peptides are acid- and base-sensitive[7] and often contain additional reactive amino acid side chain functions. Thus, their synthesis calls for the development of sophisticated protecting-group strategies employing blocking functions for carboxy, amino, thiol, and hydroxy groups, which are orthogonally stable to each other, yet can be removed selectively under very mild conditions.[8] Here we describe a protecting-group strategy for the synthesis of the characteristic C-terminal octapeptide of H-Ras in correctly modified and selectively unmasked form. The key feature of our strategy is the orchestration of the acid-labile *tert*-butyl ester function as carboxy protecting group, the Pd^0-sensitive allyloxycarbonyl (Aloc) urethane function as amino-blocking group, and the reduction-labile *tert*-butyl disulfide function for masking of thiol groups.

The C-terminal octapeptide **1a** of H-Ras contains one acid-sensitive farnesyl thioether and two base-labile thioester moieties. In addition, a serine OH group and, in particular, a lysine ε-amino group are located in the immediate vicinity of the thioester groups, increasing the danger of $S \rightarrow O$ and $S \rightarrow N$ acyl migrations in the course of the synthesis. In a retrosynthetic sense, triply lipidated peptide **1a** was divided into two selectively unmasked palmitoylated tripeptide building blocks **2a** and **3a**, N-terminally unmasked lysine derivative **4**, and *S*-farnesylated cysteine methyl ester **5** (Scheme 1). Similarly, it was planned to build up the farnesylated but nonpalmitoylated analogue **1b** by means of *S*-protected building blocks **2b** and **3b**. Octapeptide **1b** or its *S*-protected analogue might be used advantageously for the investigation of in vitro and in vivo palmitoylation[6] and the role of *S*-farnesylation.[9]



Scheme 1. *Retrosynthetic strategy for the synthesis of C-terminal H-Ras octapeptides.*

The synthesis of the *S*-acylated tripeptide **2a** (Scheme 2) started with an *N*,*N*-dicyclohexylcarbodiimide(DCC)- and *N*-hydroxysuccinimide(HOSu)-mediated coupling of Aloc-protected proline **6** with glycine and condensation of the resulting dipeptide with cystine bis(*tert*-butyl ester) to give disulfide **7**. Subsequent reduction of the disulfide bridge by treatment with dithiothreitol (DTT), *S*-acylation with palmitoyl chloride, and selective acid-mediated removal of the *tert*-butyl ester group

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