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Emission of characteristic fluorescence from the ligand–cytosine complex in U_A/ACU bulged RNA duplex

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Abstract—Here we show that *N*,*N*-bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine (DANP) binds to the single cytosine bulge in RNA duplexes. When the base pairs flanking the C-bulge were A–U base pairs, a characteristic fluorescence was emitted from the DANP–C-bulge complex. The fluorescence would be useful for detecting the C-bulge in RNA secondary structures.

1. Introduction

Bulged structures consisting of unpaired bases in RNA duplexes are found in regions important for protein–RNA recognition and for the catalytic activity of RNA nanomachines such as ribozymes.[1] Bulges affect the secondary and tertiary structures of various types of RNA and appear to be involved in many biological control processes.[2,3] Small molecules with specificity for certain nucleic acid secondary structures are useful for mapping the secondary structures of RNA and to lock the secondary structures to prevent their folding up into functional tertiary structures.[4] Considering the universal distribution of bulged structures in all types of structurally functional RNAs,[3] it is important to develop small molecules as binding and detecting agents for RNA bulged structures. Especially, fluorescent small molecules sensitive to the local environment within DNA and RNA duplexes have been envisioned to be useful probe molecules for the detection of physiologically important nucleic acid base bulges.[5] We have reported that *N*,*N*-bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine (DANP, Fig. 1) could bind not only to the single cytosine but also to the thymine bulge in duplex DNA with a 1:1 binding stoichiometry. DANP had two 3-aminopropyl side chains that enhanced the electrostatic interactions to DNA and increase the solubility to the buffer. DANP showed a 30 nm shift of an emission maximum to a longer wavelength upon its binding to the cytosine and thymine bulge DNA duplexes.[6] In order to see if DANP could bind to a single nucleotide bulge in RNA duplex and change the fluorescence as observed for DNA, we have measured the melting temperature (T_m), UV absorption, and fluorescence spectra of DANP in the presence of RNA duplexes. We here report that DANP binds to the single cytosine bulge in RNA duplexes and emits the characteristic fluorescence when the base pairs flanking the C-bulge are A–U base pairs. The data described here would be useful for the rational design of small molecules as RNA bulge binding agents.

2. Results and discussion

The binding of DANP to RNA duplexes containing a single nucleotide bulge was examined by measuring the melting temperatures (T_m) of duplexes 5′-r(CUAAC U_A AAUG)-3′/3′-r(GAUUG ANU UUAC)-5′ and 5′-r(CUAAC G_G AAUG)-3′/3′-r(GAUUG CNC UUAC)-5′containing a single nucleotide bulge N, where N was adenine, guanine, cytosine, or uridine. We chose C-bulges in two flanking sequences of (U_A/ANU) and (G_G/CNC). The increase of T_m (ΔT_m) of RNA duplexes in the presence of DANP is summarized in Tables 1 and 2.

The ΔT_m is highly dependent on the base at the single nucleotide bulge site and also on the base pairs flanking the bulge. While DANP did not stabilize the fully matched RNA duplexes, it stabilized the C- and

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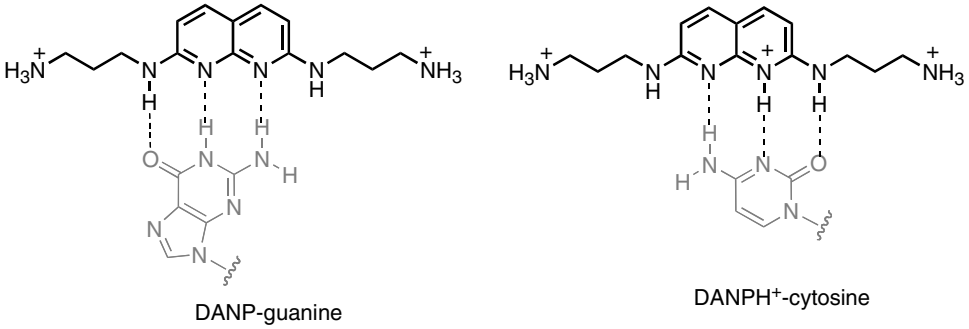

Figure 1. Structures and possible hydrogen bonds in DANP–guanine and DANPH$^+$–cytosine in the pH 7.0 solution.

Table 1. Melting temperatures of U_A/ANU RNA duplexes

N[a]	$T_m(-)$ (°C)[b]	$T_m(+)$ (°C)[c]	ΔT_m (°C)[d]
None	36.8	37.3	0.5
A	25.0	27.9	2.9
C	23.7	28.7	5.0
G	23.0	27.6	4.6
U	32.8	34.0	1.2

[a] U_A/ANU = 5′-r(CUAACU_AAAUG)-3′/3′-r(GAUUGANUUUA C)-5′containing a single nucleotide bulge (N).
[b] Melting temperatures in the absence of DANP.
[c] Melting temperatures in the presence of DANP.
[d] $T_{m(+)} - T_{m(-)}$[DANP]/[RNA] = 10/1, [RNA] = 5 μM, pH 7.0, 10 mM Na cacodylate, 100 mM NaCl.

Table 2. Melting temperatures of G_G/CNC RNA duplexes

N[a]	$T_m(-)$ (°C)[b]	$T_m(+)$ (°C)[c]	ΔT_m (°C)[d]
None	50.1	50.3	0.2
A	34.1	35.7	1.6
C	39.1	41.3	2.2
G	33.8	36.5	2.7
U	35.7	37.5	1.8

[a] G_G/CNC = 5′-r(CUAACG_GAAUG)-3′/3′-r(GAUUGCNCUUA C)-5′containing a single nucleotide bulge (N).
[b] Melting temperatures in the absence of DANP.
[c] Melting temperatures in the presence of DANP.
[d] $T_{m(+)} - T_{m(-)}$ [DANP]/[RNA] = 10/1, [RNA] = 5 μM, pH 7.0, 10 mM Na cacodylate, 100 mM NaCl.

G-bulged RNAs. In the U_A/ANU sequence, the ΔT_m decreased in the order of C-, G-, A-, and U-bulges. In the G_G/CNC sequence, the T_m of RNA ($T_{m(-)}$) was higher than that in U_A/ANU, making the ΔT_m relatively small. The largest ΔT_m (2.7 °C) was observed for G-bulge, whereas the highest T_m of 41.3 (°C) in the presence of DANP was recorded for the C-bulge. The 2,7-diamino-1,8-naphthyridine could form three hydrogen bonds to G and to C upon a protonation of nitrogen at either 1 or 8 position (Fig. 1). The higher T_m values in G_G/CNC than that in U_A/ANU suggested that DANP bound to G- and C-bulges by hydrogen bonds was further stabilized by the neighboring base pairs as we observed for the DANP binding to the single nucleotide bulge in DNA duplexes.[6] However, the ΔT_m recorded for bulged RNA duplexes were rather smaller than those observed for bulged DNA duplexes. These differences could be rationalized by the difference in the initial structures of DNA and RNA duplexes; that is, DNA duplex was in B-form, whereas RNA duplex was in A-form.

We then measured the UV absorption of DANP in the presence of RNA duplexes. The UV absorption was measured with a constant DANP (10 μM) and RNA (30 μM) duplex concentrations in sodium cacodylate buffer (pH 7.0). The UV absorption of DANP in the presence of full match or bulge-containing RNA duplexes showed characteristic spectra. The obvious difference is that the maximum absorption peak was shifted to a longer wavelength. One-main-peak at 361 nm was observed for free DANP, and 362 nm in the presence of full match RNA duplex with a weak shoulder at 380 nm. For the U-bulge RNA duplex, the shoulder peak became apparent, and for the A- and G-bulges it was another main-peak with the maximum absorption at 381 and 383 nm, respectively. For the C-bulge, the single main-peak was observed only at 381 nm. The absorption spectrum was characteristic from those with other RNA duplexes in terms of the spectral shape. The very broad absorption extended its tail to the region of over 400 nm. Considering the formation of fully matched hydrogen bonds between the protonated DANP (DANPH$^+$) and the cytosine base, the peak shift and broadening was suggested to be due to the protonation of the 1,8-naphthyridine chromophore. This assumption was supported to be the case by the UV absorption spectra in the buffer solution at pH 6.0 and 8.0.

In the condition of pH 6.0, DANP was mostly protonated according to the pK_a of 6.8 for the DANPH$^+$.[6] Compared with the UV spectra at pH 7.0, the free unbound DANPH$^+$ shows a broader two-main-peak absorption curve in pH 6.0 (Fig. 2b). DANP shifted its maximum absorption to the longer wavelength region. Accordingly, the UV spectra of DANP in the presence of A-, G-, U-bulged RNA and fully matched RNA duplexes also shifted to give the absorption maximum at about 381 nm. In contrast, the UV spectrum of DANP in the C-bulge RNA duplex was not markedly changed and the slopes of the absorption spectra over 380 nm were almost identical. In the solution of pH 8.0, the UV spectrum of all the samples showed the main-peak at 361 nm. The shoulder peak at 381 nm became considerably weaker than that in pH 7.0 solution. However, the absorption was strongest for the DNAP in the presence of C-bulge RNA, suggesting that at pH 8.0 some fraction of DANP was protonated and bound to the C-bulge. In pH 8.0, the free DANP would not be protonated as judged from the spectra, but DANP bound

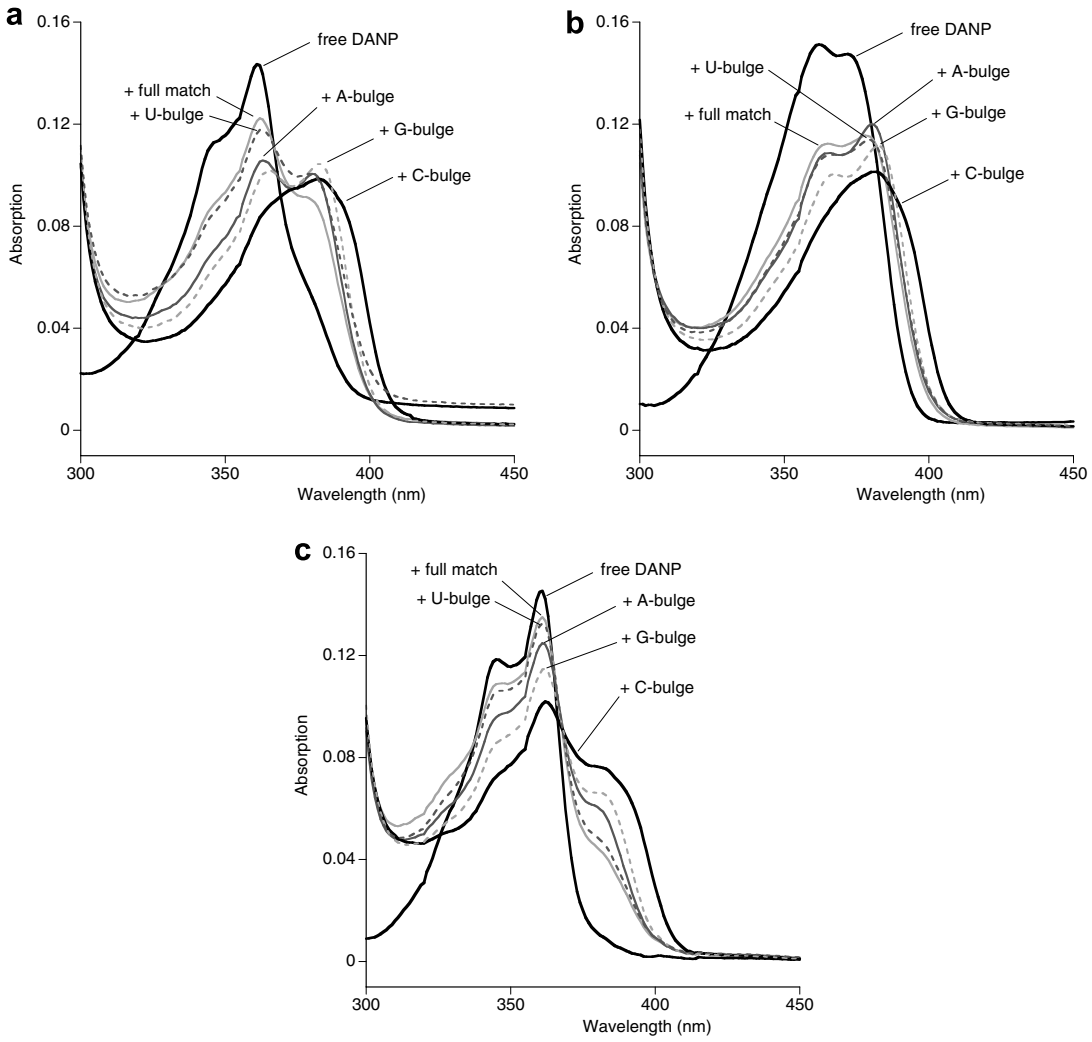

Figure 2. UV absorption spectra of DANP in the presence of RNA duplexes U_A/ANU at (a) pH 7.0, (b) pH 6.0, and (c) pH 8.0. [RNA] = 30 μM, [DANP] = 10 μM, 10 mM Na cacodylate, 100 mM NaCl.

to the C-bulge was most likely to be protonated. This is because the proton attached to DANP N1 (or N8) was further bound by cytosine N1 (*cf.* Figure 1 right). Therefore, the pK_a of the hydrogen would be much larger than that of the protonated DANP. These results suggested that DANP would be protonated when it bound to the C-bulge RNA duplex. A similar absorption change of DANP with response to the presence of the G_G/CNC bulged RNA duplexes was observed in both pH 7.0 and 6.0 buffer solutions (see Fig. 3).

We then measured the fluorescence spectra of DANP in the presence of full match and single nucleotide bulge-containing RNA duplexes. The fluorescence spectra obtained by exciting at the absorption maximum were bulge-dependent. In the presence of full match and U-bulge RNA duplexes in the U_A/ANU sequence, DANP fluorescence showed a decreased intensity without changing the shape of the spectra, suggesting that DANP had weak interactions to these RNA duplexes (Fig. 4a). These observations were in good agreements with the small changes in UV spectra. In the presence of A-, G-, and C-bulge RNA duplexes, the spectra were broad and different from that in the presence of U-bulge RNA duplex. Among these spectra, the fluorescence of DANP bound to the C-bulge showed a long emission tail with a higher intensity compared to those observed for G- and A-bulges. When these DANP solutions were excited at 400 nm, intense fluorescence was selectively observed for the DANP bound to the C-bulge in the U_A/ACU sequence (Fig. 4b). Because DANP bound to these RNA has very weak absorption at 400 nm, only modest signals were observed for free DANP and DANP bound to other bulges, indicating the fluorescence could be detected even in the presence of free DANP. This characteristic fluorescence was highly dependent on the base pairs flanking the C-bulge. The fluorescence of DANP bound to the C-bulge in the G_G/CCC sequence was totally quenched by the guanines in the neighboring G–C base pairs (Fig. 5). It is important to note that the DANP fluorescence was effectively quenched by the G in the neighboring G–C base pair but not by the G of the bulged site as evidenced by the DANP fluorescence with the U_A/AGU duplex. Furthermore, DANP fluorescence was quenched in the presence of U-, G-, and C-bulges in G_G/CNC sequence. These observations suggested that DANP binding to the bulged sites in G_G/CNC was pronounced by both hydrogen bonds

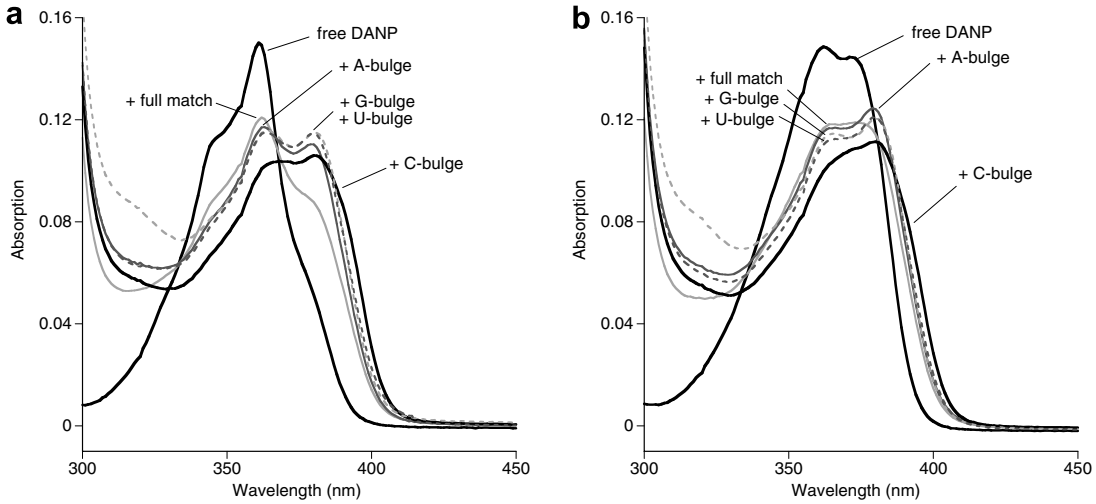

Figure 3. UV absorption spectra of DANP in the presence of RNA duplexes G_G/CNC at (a) pH 7.0 and (b) pH 6.0. Solution conditions are the same as in Fig. 2.

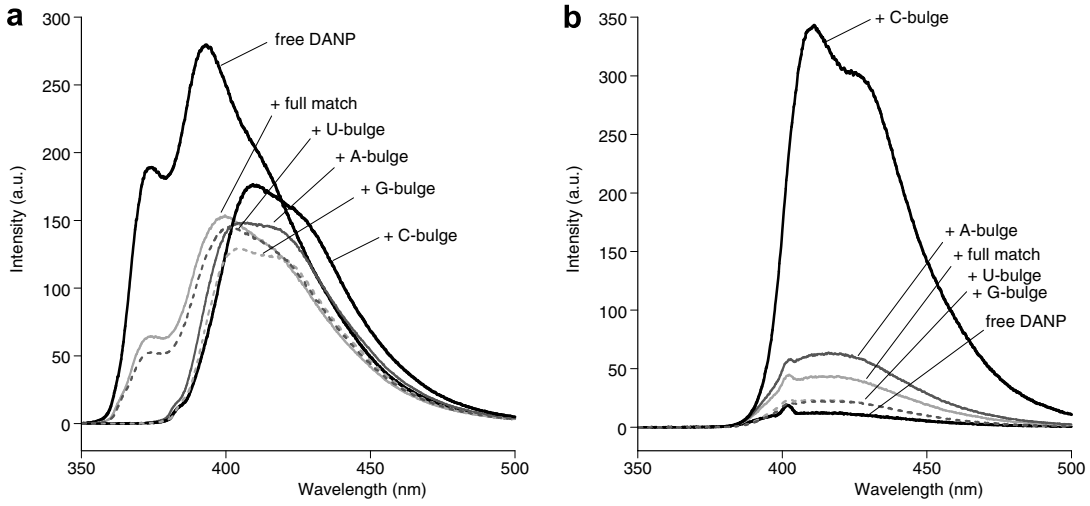

Figure 4. Fluorescence spectra of DANP in the presence of RNA duplexe U_A/ANU at pH 7.0. Spectra were obtained by an excitation (a) at the maximum UV absorption wavelength and (b) at 400 nm. Solution conditions are the same as in Figure 2.

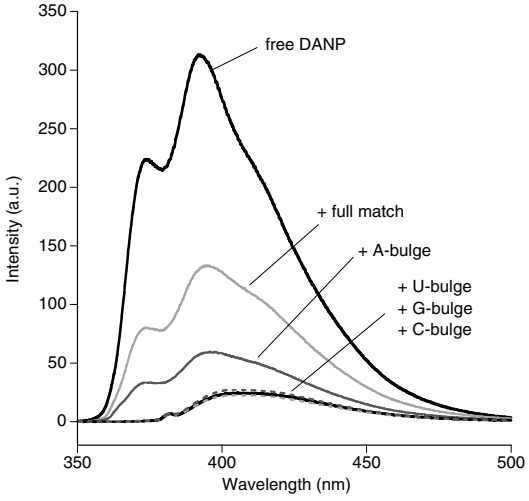

Figure 5. Fluorescence spectra of DANP in the presence of RNA duplexe G_G/CNC at pH 7.0. Spectra were obtained by an excitation at the maximum UV absorption wavelength. Solution conditions are the same as in Figure 2.

and the stacking by the guanines. The characteristic fluorescence of DANP bound to the U_A/ACU was most likely due to the stacking interactions of the neighboring A to DANP bound to the C-bulge. The proposed structure of DANP–C bulge complex in the U_A/ACU sequence was simulated by molecular modeling analysis (Fig. 6). The simulation supported the stacking of DANP by the neighboring adenines.

3. Conclusion

From the data of UV-melting, absorption, and fluorescence spectra, DANP was found to bind to G- and C-bulge RNA duplexes. When DANP bound to the C-bulge RNA duplex in the U_A/ANU sequence, the characteristic fluorescence was emitted from the DANP–C bulge complex. This fluorescence could be detected even in the presence of free unbound DANP. This spectroscopic property suggested that DANP could be used as an imaging tool for the U_A/ACU in the

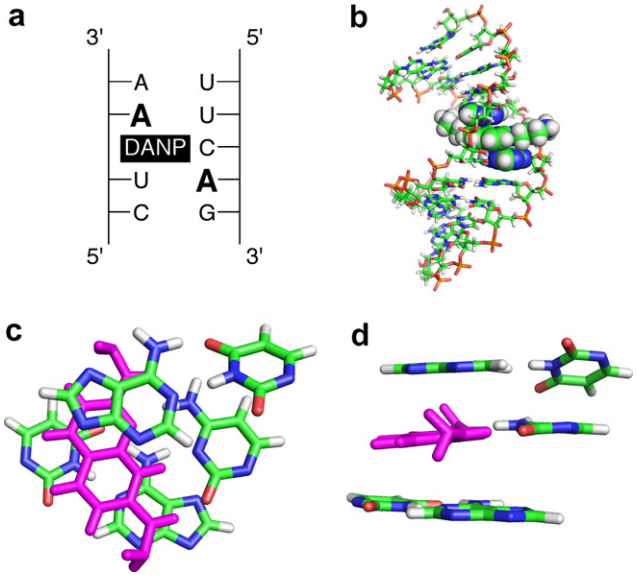

Figure 6. (a) An illustration of the DANP-C bulge complex in RNA duplex. Two adenines stacked with DANP are shown in a bold face. (b) A structure obtained by molecular modeling simulation by MacroModel (ver. 9.1). DANP and two adenines in the U_A/ACU sequence are shown by a space filling model. (c,d) Stacking of DANP (magenta) with the flanking base pairs. The alkyl side chains of DANP, phosphate backbone, and sugar parts are omitted for clarity.

RNA secondary structure. Further studies on the DANP binding would be useful for the design of small molecules detecting the important RNA secondary structures.

4. Experimental

4.1. Measurements of melting temperature of bulge-containing RNA duplexes

All RNAs were purchased from Hokkaido System Science, Japan, and purified by desaltation. DANP (50 μM) was dissolved in a sodium cacodylate buffer (10 mM, pH 7.0) containing RNA duplexes (5 μM) and sodium chloride (100 mM). The mixture was cooled slowly to 0 °C and kept at 0 °C for 15 min to make sure that the starting oligomer was in the duplex state. The thermal denaturation profile was recorded on a SHIMADZU UV2550 spectrometer equipped with a SHIMADZU TMSPC-8 temperature controller. The absorbance of the sample was monitored at 260 nm from 0 to 85 °C with a heating rate of 1 °C/min.

4.2. UV and fluorescence spectra measurements

UV spectra were recorded on a SHIMADZU UV2550 spectrometer. Fluorescence spectra were recorded on a SHIMADZU RF-5300PC. RNA samples were prepared in 10 mM sodium cacodylate buffer at the designate pH in the presence of 100 mM sodium chloride. Excitation wavelength for the fluorescent measurements was the wavelength with the maximum absorption unless otherwise noted.

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