The acridine ring selectively intercalated into a DNA helix at various types of abasic sites: double strand formation and photophysical properties

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ABSTRACT

The interactions between the intercalating agent and the three types of abasic sites: abasic frameshift, apurinic and apyrimidinic, were investigated. 9-amino-6-chloro-2-methoxyacridine (ACMA), whose spectroscopic properties are strongly perturbed by the environment, was selected as the intercalating agent. The optically pure threoninol derived from the reduction of L-threonine was used as an artificial abasic site mimicking the ring-opened natural ribose. In order to secure the selective intercalation to the adjacent abasic site, ACMA and the abasic site were connected through a tri- pentamethylene linker. These modified oligonucleotides covalently linked to an ACMA molecule at the internucleotide site having the same base-sequence were synthesized using the acridine-phosphoramidites. Although all the modified oligonucleotides lack a nucleobase at the intervening position, these double strands showed high thermal stability. The pentamethylene linker and the apyrimidinic systems were especially stabilized. At the same time, sharpness of the absorption spectra and a new fluorescent band of the acridine, due to the fixation of the environment around ACMA, were observed. Therefore, it is concluded that the acridine binds preferentially to the apyrimidinic site rather than the frameshift abasic site and that the surroundings of the acridine are strictly fixed at the microenvironmental level.

INTRODUCTION

The chemical modification of oligonucleotides with pyrene, psoralene, fluorescein, etc., has gained considerable importance in recent years (1,2). This is mainly due to its potential application to biological tools such as antisense, triple helices, DNA probes or ribozymes. In this field, 9-aminoacridine or its derivatives-conjugated oligonucleotides have been widely used as in the pioneering work of Hélène et al. (3–5). In these reports, the 9-aminoacridine derivative has been covalently connected to the defined sequence at 5'-phosphate through a linker chain with various lengths, and these modified oligonucleotides have been examined with respect to the thermodynamical properties in the form of a double helix with poly(rA) or a triple helix with native DNA. It has been concluded that the pentamethylene-linker length was the most suitable to stabilize the connecting DNA helix, whereas there were at least two binding sites of the acridine, that is, at the end of the helix and into the neighboring base stacking. On the other hand, when these oligonucleotides are used as the probes such as in DNA sequencing, it is obvious that these sensitivities and accuracy will be strongly dependent on the degree of fixation of these dyes into the target position of DNA. Because the 3’ or 5’ end of DNA are thermodynamically fluctuated, the stacking of nucleobases is unstable and the binding site of the dye could not be defined. Moreover, for a systematic investigation of electron transfer in DNA helix (6–8), more careful design to fix the donor and acceptor molecules will be required.

In order to selectively intercalate the acridine dye, we have already reported a methodology for replacement of a nucleoside with an acridine dye at the internucleotide site (9), in which the acridine was linked through a pentamethylene linker as shown in Figure 1. It was also shown that the acridine ring may be selectively intercalated into the adjacent artificial abasic site. Although we carefully set the linker length to pentamethylene based on a molecular model, further investigation of the length for strict fixation of the acridine into the DNA helix is needed. In general, a shorter linker will interfere with the effective stacking between the acridine and the neighboring base. On the other hand, a longer linker will permit effective stacking, but it becomes necessary for the acridine ring to bind to the other sites. Moreover, the base-sequence around the acridine will be important factors (11).

In this report, we study the thermal stability and photophysical property of duplexes consisting of oligonucleotides containing 9-aminoacridine derivatives via various lengths in one strand and also various types of a complementary strand with a ‘bulge’ nucleobase opposite the acridine ring.

MATERIALS AND METHODS

High performance liquid chromatography (HPLC) was made to run on a Shimadzu LC/CTO-6A model equipped with a Waters 991J 3D-UV detector, using a reverse-phase COSMOSIL
Figure 1. Structural drawing of an ACMA-modified oligonucleotide at intervening artificial abasic site through a trimethylene linker.

AR-300 column (4.6 × 150 mm). Absorption spectra and thermal stability measurements were recorded on a Shimadzu UV-2200 spectrophotometer with a theromoelectrical-cell holder. Fluorescence spectra were recorded on a Shimadzu RF-503A spectrophotometer. The measurements were carried out in 1 cm quartz cell at 0°C. The fluorescence quantum yields (Φf) were determined by comparison with the reference solution R using the following equation: (12)

Φ = ΦR(n/nR)²•(D/R)•(A/A R)

where n, D and A are the solvent refractive index, absorbance of the reference solution at the chosen excitation wavelength and the area of the corrected emission spectrum plotted on a wavenumber scale, respectively. The subscript R stands for the reference solution. 9-Amino-6-chloro-2-methoxyacridine in a 10 mM phosphate buffer and 0.15 M sodium chloride (pH 6) containing 1% ethanol was used as the reference solution [Φf = 0.80 (13)].

The extinction coefficient at 260 nm of acridine-modified oligonucleotides was determined by dividing the oligonucleotides into three areas, 5′-(7mer–acridine–7mer)-3′; these 7mers were calculated by the nearest-neighbor methods (14) and that of the acridine was 2.0 × 10⁴/M/cm determined by the differential absorption spectra method (15). Thus, the extinction coefficients of the modified oligonucleotides 3–5 were calculated to 1.81 × 10⁴/M/cm, although their linker lengths are different. The mixing curves monitored at 260 nm of acridine-modified oligonucleotides 3–5 with complementary sequence n–a satisfied these results (10).

The hypochromic effect is quantitatively expressed by the percent hypochromism (% H). It was calculated according to the following equations.

\[ \Delta = \int \varepsilon(\nu) \, d\nu \]

\[ \text{H}\% = 1 - \Delta b/\Delta f \]

where ε(ν) stands for the extinction coefficient of acridine at defined wavenumber. The calculation was carried out in the range responsible for the S₀ → S₁ transition (370–500 nm) as shown in Figure 4. Ab and Af signify the extinction coefficient of acridine-modified oligonucleotides and free acridine [quinacrine (16)], respectively. The measurements were carried out at 0°C in pH 7.2 phosphate buffer with 150 mM sodium chloride.

**Synthesis of oligonucleotides covalently linked with the acridine ring via a trimethylene to pentamethylene linker**

In order to introduce the acridine moiety at the intervening site of oligonucleotide, phosphoramidites were synthesized in our previous study (10). These reagents were dissolved in dry acetonitrile at 0.15 M and fed to ABI PCR-mate 391 DNA synthesizer and then used in the standard synthetic cycle with an extended coupling time (5 min) during the phosphoramidite coupling. Expedite amidite (Millipore) was used as natural deoxyribonucleotide–phosphoramidites to perform the deprotection of the adenine base under mild conditions (conc. ammonia, 2 h at room temperature). The 5′-dimethoxytrityl substrate was retained to facilitate the purification. Figure 2 shows the HPLC chart of 5′-dimethoxytritylated 5 (see below) just after the treatment with conc. ammonia. Then the 5′-dimethoxytrityl substrate was removed with 80% acetic acid (15 min). Finally, the

Figure 2. HPLC elution profiles of 5′-dimethoxytritylated DNA oligomer, sequence 5 immediately after the treatment with conc. ammonia. Chromatography was carried out with a linear gradient of acetonitrile versus 0.1 M triethylammonium acetate (pH 7.0) ranging from 6 to 60% over 30 min at a flow rate of 0.8 ml/min on Cosmosil C-18 column (150 × 4.5 mm), detected at 260 nm. Inset: absorption spectra of finally purified DNA oligomer, sequence 5 in distilled water.
oligonucleotides were purified with the reversed-phase HPLC. These modified oligonucleotides showed absorption spectra indicating an acridine ring tethered to a 14mer oligonucleotide from each extinction coefficient (Fig. 2, inset).

The following three oligonucleotides, 3–5, were synthesized, where [Acrn] means acridine tethered via a (CH₂)ₙ length linker chain (Fig. 1). Then the three types of the complementary oligonucleotides n, t and a, that is, normal, thymine and adenine opposite the acridine, respectively, were also synthesized.

3: 5'-ATAATAA[Acr3]AATTAAT
4: 5'-ATAATAA[Acr4]AATTAAT
5: 5'-ATAATAA[Acr5]AATTAAT
n: 5'-ATTAATTATTAT
  t: 5'-ATTAATTATTTAT
  a: 5'-ATTAATTATTTAT

In the following sections, the double strand between acridine–oligonucleotide and the complementary one is represented like 3n or 4a.

RESULTS

We have measured the absorption spectra, thermal stabilities and fluorescence properties of various duplexes of acridine-modified 14mer 3–5 with the complementary sequence n, t and a. All the results are listed in Table 1 and graphically illustrated in Figure 3 to facilitate the evaluation of dependence on the linker length and the presence of the nucleobase opposite the acridine ring.

<table>
<thead>
<tr>
<th>Acr-ODN</th>
<th>Single strand</th>
<th>Complementary ODNs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypo / %ᵃ</td>
<td>n</td>
</tr>
<tr>
<td>3</td>
<td>Tₘ / °Cᵇ</td>
<td>23</td>
</tr>
<tr>
<td>Fluorescence Q. Y.ᶜ</td>
<td>0.41</td>
<td>0.55</td>
</tr>
<tr>
<td>Hypo / %ᵃ</td>
<td>26</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>Tₘ / °Cᵇ</td>
<td>–</td>
</tr>
<tr>
<td>Fluorescence Q. Y.ᶜ</td>
<td>0.36</td>
<td>0.57</td>
</tr>
<tr>
<td>Hypo / %ᵃ</td>
<td>25</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>Tₘ / °Cᵇ</td>
<td>–</td>
</tr>
<tr>
<td>Fluorescence Q. Y.ᶜ</td>
<td>0.35</td>
<td>0.39</td>
</tr>
</tbody>
</table>

All measurements were carried out in 10 mM phosphate buffer–150 mM sodium chloride (pH 7.2).

ᵃCompared with free acridine (quinacrine) in the same buffer.
ᶜMeasurements were carried out at 0°C.
ᵇTₘ values were determined at 2.5 µM total strand concentration.

UV absorption spectra

In all the stranded forms, a strong hypochromic effect in the region of the absorption of the acridine ring was detected as shown in Table 1. In these systems, since an acridine ring interacts with its connecting single-stranded nucleobase, these absorption maxima were slightly red-shifted even in the single stranded form (at ~427 and 451 nm) compared with free acridine (15) (424 and 445 nm, respectively). Differences in the maximum wavelength were <2 nm in all the single and double strands. In several cases, the hypochromic effect of the single strand is stronger than that of the duplex. In general, the hypochromic effect refers to the stacking interaction between the dye and
nucleobase (15). The acridine ring in a single strand can be stacked to all the tethering nucleobases in the most favorable conformation, because the structure of the single strand is rather flexible. When the double helices are formed, the stacking sites in the duplex can be restricted to nucleobases around the AP site. Hence, the hypochromicity varied from the corresponding single strands depending on the linker length and the base-sequence (17), and comparison of the hypochromicity of the acridine-modified single strand with that of the duplex is not appropriate in considering the degree of stacking. When acridine-modified DNA 3–5 bound to the n sequence, the hypochromic effect increased in the order of 5n > 4n > 3n (Fig. 3A). It is noteworthy that a new weak absorption band appeared at around 475 nm, although 5n systems showed the strongest hypochromic effect in all the double strands. This band was due to the presence of the other binding sites to the neighboring regular A•T base stacking (5). Therefore, this longer chain allowed not only the effective stacking with the nucleobase in both sides of the abasic site but also the intercalation to the other side(s). On binding to bulge systems t and a, hypochromic effects decreased as shown in Figure 3A. Especially, 3 and 5 were strongly affected by introduction of the nucleobase at the opposite side of the acridine ring. The maximum hypochromic effect was shown in 4 that tethered with a middle length linker. In these 4t and 4a systems, sharpness of the absorption band was simultaneously detected which indicates that the acridine ring is surrounded with a rigid matrix such as crystal (Fig. 4). This behavior is quite different from the other dye–DNA conjugate systems (5,18,19).

**Thermal stability**

To evaluate the effects of introducing the acridine ring and nucleobase at its opposite side, the thermal stability of the whole double strand was measured. All the double strands showed clear sigmoidal curves like 5n, t and a monitored at 260 nm (Fig. 5), signifying the double-stranded structure remains. The absorbance of the acridine moiety detected at 427 nm changed in the same manner, so the acridine binds to the DNA helix cooperatively (9,10). Similarly to the measurement of absorption spectra, double-stranded forms with an n-type sequence were most stable in the order of 3n > 4n > 5n (Fig. 3B). In bulged systems, although decrease in thermal stability of 3t, a and 5t, a was much more pronounced than that of the n system, double strands 4t, a showed particular stability compared with the longer or shorter linker systems. These results are closely similar in the manner of hypochromic effects (see Fig. 3A and B) showing that the 4 systems especially stabilize the DNA structures in the bulge systems.

**Steady-state fluorescence**

It is well known that the fluorescence of 9-amino-3-chloro-6-methoxyacridine (ACMA) is enhanced by intercalation to DNA consisting of adenine and thymine, and quenched only with guanine (20). Since the synthesized oligonucleotides 3–5 consisted of only deoxyadenosine and deoxythymidine, the fluorescence would be enhanced. The intensity of the fluorescence would be influenced simultaneously by the style of intercalation and thermodynamical stability around the acridine ring. All the acridines in double helix emitted strong fluorescence (Fig. 6A–C).

Under the double strand conditions, the fluorescence quantum yields increased in the order of a > t > n (Fig. 3C). This order is the reverse of that of melting temperatures. These results mean that the fluorescence intensity of the acridine ring does not necessarily reflect thermal stability of the whole DNA structure.
The fluorescence intensity depends on the microenvironment around the acridine ring as discussed below.

The fluorescence quantum yields of 5 series were somewhat weaker than those of other double strands (see Table 1 and Fig. 3C). These results are consistent with the finding that the linker length of 5 is too long to fix the acridine into the desired site. Since the flexibility of or around the fluorescent dye increases the rate constant of the non-radiative decay process, a loosely fixed dye emits lower fluorescence (21).

On the other hand, the 4 series gave the strongest emission. Moreover, a new emission band around 475 nm clearly appeared in the 4t and a systems (Fig. 6B), which forms a complete mirror image of the absorption spectra (Fig. 4). These results can be explained by the two-state model in the excited state of ACMA (13). The surrounding solvent of the monocationic ACMA molecules gives rise to strong solvation due to the charge–dipole interactions and the relaxation of the solvent is very slow. Hence, the lowest excited state of ACMA consequently can be divided into two states, P* and R*; only one species P can be directly photoexcited from the ground state to P* and relaxation of the solvent gives the lower energy state R* (Scheme I). The new emission band around 475 nm can be assigned to one from the P* state (13). In general, since the rate constant of the relaxation k3 is much larger than that of the deactivation process from P*, this new band was only detected in conditions where the relaxation was slower, e.g., use of a viscous organic solvent like glycerol as well as at low temperature. These new bands had never been detected in any inter- or intramolecular intercalation between ACMA and DNA, whereas they have been reported in many ACMA–DNA or RNA systems (17,20,22,23). Therefore, it is considered that the relaxation of the microenvironment around the ACMA in 5t and 5a is fixed in the excited states.

These results are consistent with the above spectroscopic and thermodynamical findings that a middle-length linker chain is most suitable to fix the acridine ring into the DNA helix selectively. Although the presence of a bulged nucleobase in the opposite side interferes with the intercalation of the acridine ring in 3 and 5, it assists the fixation of the acridine ring firmly in 4 having a middle length chain.

**DISCUSSION**

The data provided here clearly indicate that the way of intercalation of acridine into the neighboring abasic site depends on the linker length and the base sequence. The microenvironment of the acridine ring was also influenced by these factors.

Except for the acridine and linker moiety, double helices DNA consisting of 3–5 and the complementary sequence n, t and a can be considered as conventional abasic DNA: abasic frameshift, apurinic and apyrimidinic site (AP site), respectively. Introduction of an AP site into 13mer DNA decreased the melting temperature of the duplexes by ∼19°C (24). On the other hand, the abasic frameshift in 15mer DNA caused a rather small decrease, that is, ∼10°C (9). These numerical values cannot be directly compared, because these sequences and conditions are different. However, a certain trend is evident: in abasic frameshift DNA, the nucleobases on either side of the abasic site can be stacked over each other compensating the lack of nucleobase and the abasic site is rotated out of the helix (25). In the AP site of DNA, the nucleobases at opposite sides of the abasic site are in an equilibrium between the inside (intrahelical) and the outside (extrahelical) of the helix (26). Although the nucleobase prefers the intrahelical form, the difference in those two conformational energies is relatively small. These thermal stabilities can be ordered, that is, native > abasic frameshift > AP site DNA.

Conjugation of ACMA to these abasic sites, oligomer 3–5, increased the thermal stabilities. The melting temperatures of the n series were higher than those of the t and a series. However, taking into account the differences in these abasic styles as described above, 4t and 4a are stabilized rather than 4n from each abasic style.

The interactions between intercalator and DNA lesion such as the abasic site have been investigated. The ‘true’ apurinic site was strongly interacted with 9-aminooellipticine (27) and rapidly cleaved with this intercalator (24). Moreover, 9-aminooellipticine covalently linked to the true abasic site through a reduced Schiff form was synthesized and the structure was evaluated by 2D-NMR (28). The 9-aminooellipticine was inserted into the abasic site avoiding the opposite thymine. It was also shown that ethidium bromide had an increased affinity with an oligonucleotide containing a bulged cytosine compared with a normal duplex of the same sequence (29) and 9-aminocarboxylic acid also preferentially bound to the bulged site (30). Although this system is not identical with ours, it is similar in that the DNA lesion site such as abasic or bulge in the double helix acts as an anchoring site for a dye.

Intercalation to a regular DNA requires the spreading of local-base stacking before insertion of the dye into the DNA. This energy, of course, would be compensated by the newly emerged stacking between the dye and both sides of the base pairs (29). In the abasic oligonucleotide, this spreading energy is considerably small since the stacking is absent in the abasic site. The intercalation to the abasic site, therefore, would show higher stability compared with that to regular DNA.

9-Aminoacridine derivatives intercalate to a DNA helix so as to maximize the stacking area (31). Thus, the most favourable orientation is that in which the longer axis of the acridine is parallel to the stacked base pairs in DNA. Connection of the intercalator to the DNA forces the number of the binding sites of the intercalator to decrease, but the presence of the linker would preclude the intercalation like these free intermolecular intercalations. Hence, investigation of the linker length is important. Now we can conclude the tetramethylene linker is the most suitable for the present purposes.
We consider that the acridine ring in 3n and 4n intercalates into the adjacent abasic site illustrated as I in Scheme II. This structure is similar to that in the abasic frameshift systems (25) in which the acridine (model AP site) interferes with the neighboring nucleobases stacked on each other. Presence of a bulged base at the opposite side of the acridine ring (model AP site) interferes with nucleobases that are stacked on each other. Presence of a bulged base outside of the helix and both the neighboring nucleobases are stacked on each other. Therefore, it can be reasonably considered that the bulged thymine or adenine interact with the acridine ring and the whole DNA structure would be stabilized. However, (i) the properties of the 4t, a system were quite similar to each other, (ii) the size and hydrogen-bonding ability of these two nucleobases were considerably different. Therefore, it can be rationally considered that the bulged nucleobase was pushed out from the helix stacking. As described above, a pyrimidine or purine base opposite an AP site can be directed to either an intrahelical or extrahelical conformation. This is consistent with our consideration that the adenine or thymine base has an extrahelical conformation (Scheme II, IIb) in the 4t, a systems. For confirmation of these structures, two-dimensional NMR analysis will be appropriate and such study is now in progress.

REFERENCES