Photocontrol of DNA Duplex Formation by Using Azobenzene-Bearing Oligonucleotides

Hiroyuki Asanuma, Xingguo Liang, Takayuki Yoshida, and Makoto Komiyama*[a]

The duplex-forming activities of oligonucleotides can be photomodulated by incorporation of an azobenzene unit. Upon isomerizing the trans-azobenzene to the cis form by irradiation with UV light, the Tm value of the duplex (with the complementary DNA) is lowered so that the duplex is dissociated. The duplex is formed again when the cis-azobenzene is converted to the trans-azobenzene by irradiation with visible light. The photoregulation is successful irrespective of the position of the azobenzene unit in the oligonucleotides. The trans-azobenzene in the oligonucleotides intercalates between two DNA base pairs in the duplexes and stabilizes them because of a favorable enthalpy change. The nonplanar structure of a cis-azobenzene is unfavorable for such an interaction. These photoresponsive oligonucleotides are promising candidates for the regulation of various bioreactions.

Introduction

Recently, much interest has been given to the artificial control of bioreactions.[1] One of the most important applications is the regulation of the expression of a specific gene by antisense and antigene strategies.[2] Various types of functionalized oligonucleotides have been prepared for these purposes.[3] However, there have been no reports on modified oligonucleotides that alter the duplex-forming activity in response to photostimuli and are applicable to the photoregulation of bioreactions. These compounds should be valuable tools for biotechnology, molecular biology, therapy, and other applications.

In a preliminary communication,[4] the authors incorporated a photosensitive azobenzene into the side chain of oligonucleotides (see Scheme 1).[5, 6] Upon irradiation with either UV light or visible light, the azobenzene unit undergoes cis–trans isomerization, and accordingly the duplex-forming activities of the modified oligonucleotides (with respect to their complementary counterparts) are reversibly modulated.[7] By using these modified oligonucleotides, double-stranded DNA can be converted to two single strands (and vice versa) at a predetermined place and at a desired timing.

In the present work we have studied the photoregulation of duplex formation in more detail. By thermodynamic and spectroscopic analyses, the following points are clarified: 1) How does the position of azobenzene unit in the oligonucleotides affect the photoregulating activity?; 2) which of the thermodynamic parameters (the enthalpy change and the entropy change) governs this photoregulation?; and 3) what is the mechanism of the photo-regulation? Applications of these modified oligonucleotides to the photocontrol of enzymatic reactions are discussed.

![Scheme 1. Structures of azobenzene-modified oligonucleotides (top) and sequences of the oligonucleotides used in this study (bottom).](image)
Results and Discussion

Photoinduced change of $T_m$ values for oligonucleotide duplexes

Typical melting curves for the duplex formation between the azobenzene-modified oligonucleotide $A_8XAX_4(P)$ and $T_8$, the oligonucleotide with the complementary sequence, are shown in Figure 1. Before the photoirradiation, the azobenzene unit in $A_8XAX_4(P)$ is overwhelmingly (about 90%) present in the trans form, as confirmed by HPLC analysis. Consistently, the solution has a strong absorption around 350 nm (assignable to trans-azobenzene), and the absorbance around 440 nm (for cis-azobenzene) is virtually zero. Under these conditions, the melting temperature ($T_m$) of the $A_8XAX_4(P)/T_8$ duplex is 24.8°C (black line in Figure 1). This value is close to that (23.7°C) of the unmodified $A_8/T_8$ duplex. Upon irradiation with UV light (300 < $\lambda < 400$ nm), the trans-azobenzene residue is promptly isomerized to the cis form (irradiation is carried out for 30 min). Concurrently, the $T_m$ value of the duplex is lowered to 15.9°C (gray line in Figure 1). The change in $T_m$ ($\Delta T_m$), induced by the trans $\rightarrow$ cis isomerization, is 8.9°C. Still greater values of $\Delta T_m$ are obtained for other duplexes (Table 1).

![Figure 1. Melting curves of the trans-$A_8XAX_4(P)/T_8$ duplex (black line) and the cis-$A_8XAX_4(P)/T_8$ duplex (gray line).](image)

<table>
<thead>
<tr>
<th>Duplex</th>
<th>Polar fraction (P)</th>
<th>$T_m$ [°C]</th>
<th>$\Delta T_m$ [°C]</th>
<th>Less polar fraction (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_8/T_8$ (native)</td>
<td>35.5</td>
<td>31.3</td>
<td>23.3</td>
<td>8.0</td>
</tr>
<tr>
<td>$A_8XAX_4/T_8$</td>
<td>37.2</td>
<td>29.6</td>
<td>23.2</td>
<td>12.1</td>
</tr>
<tr>
<td>$A_8XXA_8/T_8$</td>
<td>35.8</td>
<td>29.6</td>
<td>23.2</td>
<td>12.1</td>
</tr>
<tr>
<td>$A_8XAX_4/T_8$</td>
<td>37.2</td>
<td>29.6</td>
<td>23.2</td>
<td>12.1</td>
</tr>
<tr>
<td>$T_8XT_8/A_8$</td>
<td>35.8</td>
<td>29.6</td>
<td>23.2</td>
<td>12.1</td>
</tr>
<tr>
<td>$G_3XG_4/C_6$</td>
<td>30.2</td>
<td>23.0</td>
<td>20.6</td>
<td>15.8</td>
</tr>
</tbody>
</table>

When the mixture is further irradiated with visible light ($\lambda > 400$ nm), the cis-azobenzene unit is isomerized to the trans form. The melting curve of the resultant solution is virtually superimposable on that observed before the first UV irradiation. The duplex-forming activity of the oligonucleotide has been reversibly modulated by the photoirradiation.

Effect of azobenzene unit position in the oligonucleotides on the photoregulation

The $T_m$ values for various duplexes are summarized in Table 1. From these results, the following conclusions can be drawn:

1. In both A-T and G-C duplexes, the $T_m$ value is notably changed by the photoisomerization of the azobenzene unit. The range of $\Delta T_m$ is 5–23°C for the duplexes studied here.
2. Without exception, the duplexes containing a trans-azobenzene unit are more stable than those containing a cis-azobenzene unit.
3. An azobenzene unit near the 5′ end of the oligonucleotide stabilizes the duplex (for both the cis form and the trans form). However, the stabilization is inefficient when the azobenzene unit is placed in the middle of the duplex.

These effects are especially prominent in the duplex formation by the oligo(T)s bearing an azobenzene group (see Table 1). When the position of the azobenzene group is systematically changed from the 5′ end of the oligonucleotide to its middle ($XT_7$, $TXT_6$, $T_7XT_5$, and $T_8XT_4$), the $T_m$ values of the respective duplexes monotonically decrease. Significantly, the decrease in the $T_m$ value for the cis isomers (from 26.9°C to 3°C) is far more

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was born in Utsunomiya, Japan, in 1947. After graduating from the University of Tokyo, he entered the Graduate School of this university and obtained his Ph.D. in 1975. He then worked as a postdoctoral fellow with Prof. Myron L. Bender at Northwestern University in Evanston, Illinois (USA). In 1979 he returned to the University of Tokyo as an Assistant Professor. In 1987 he became an Associate Professor at the University of Tsukuba, and, since 1992, has been a Full Professor at the University of Tokyo. Only recently he moved to the Research Center for Advanced Science and Technology of this university. His research interests include bioorganic and bioinorganic chemistry (especially DNA and RNA hydrolysis), host–guest chemistry (cycloextrins), functional polymers, and others. He was awarded with an Award for Young Scientists from the Chemical Society of Japan, a Japan IBM Science Award, an Award from the Rare Earth Society of Japan, the Inoue Prize for Science, and the Award of the Society of Polymer Science, Japan.

[a] Members of the Editorial Advisory Board will be introduced to the readers with their first manuscript.
drastic than that for the trans isomers (from 37.2 to 20.2 °C). As a result, the greatest ΔTm value (23.2 °C) is observed for the T3TX8/A8 duplex.[10]

**Tm differences between the diastereomers of modified oligonucleotides**

The ΔTm values for the P diastereomers are always greater than the corresponding values for the L isomers (see Table 1). For the T3TX8(P)/A8 duplex, ΔTm = 23.2 °C, whereas for the T3TX8(L)/A8 duplex is 12.1 °C. These differences in ΔTm mainly stem from the fact that the P isomers containing trans-azobenzene units provide more stable duplexes than do the L isomers: The Tm value of the trans-T3TX8(P)/A8 duplex (20.2 °C) is by 11.1 °C higher than that of the trans-T3TX8(L)/A8 duplex (9.1 °C). With the oligonucleotides bearing cis-azobenzene groups, however, the difference in Tm between the P and the L isomers is only within 2.4 °C. These effects are remarkable, especially when the azobenzene unit is attached to a position in the middle of the oligonucleotide.[12]

**Photocontrol of the formation/dissociation of DNA duplexes**

The formation of the A8XA8(T)/T8 duplex and its dissociation are satisfactorily photocontrolled (Figure 2). The temperature was kept constant at 20 °C, and the absorbance at 260 nm before the photirradiation was taken as the reference. For these measurements, neither temperature, pH, ionic strength, or other factors were changed. Under these conditions, the A8XA8(P)/T8 duplex is efficiently formed (Tm = 24.8 °C for the trans isomer of A8XA8(P)). Upon irradiation with UV light, the absorbance at 260 nm rapidly increases (open circle). The duplex is largely dissociated into two single-stranded oligonucleotides, and the hypochromicity due to duplex formation disappears (Tm = 15.9 °C for the cis isomer). Next, visible light is applied, and the absorbance decreases to the value before the UV irradiation (closed circle). These changes are reversibly repeated without apparent deterioration.

Figure 2. Photoinduced change of the absorbance at 260 nm of a solution containing A8XA8(P)/T8. Irradiation with either UV or visible light was carried out for 20 min at the positions indicated by arrows. Conditions: 20 °C, pH 7.1, 10 mmol dm⁻³ phosphate buffer, [NaCl] = 1 mol dm⁻³, [A8XA8(P)] = [T8] = 50 μmol dm⁻³.

Thermodynamic parameters for duplex formation of modified oligonucleotides

For all the A8XA8(T)/T8 duplexes listed in Table 2, the exothermicity (ΔH°) for the modified oligonucleotides bearing trans-azobenzene units exceeds that for the oligonucleotides with cis-azobenzene units. The differences are 56 kJ mol⁻¹ for the

<table>
<thead>
<tr>
<th>Duplex</th>
<th>A8XA8(P)/T8</th>
<th>A8XA8(L)/T8</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔH° [kJ mol⁻¹]</td>
<td>22.8</td>
<td>30.6</td>
</tr>
<tr>
<td>ΔS° [J mol⁻¹ K⁻¹]</td>
<td>139</td>
<td>128</td>
</tr>
</tbody>
</table>

[a] At T = 298 K.

Figure 3. The dependencies of (A) the exothermicity (ΔH°) and (B) TΔS° (T = 298 K) on Tm for the formation of A8XA8(P)/T8 duplexes; the open circles represent data for A8XA8 oligonucleotides bearing trans-azobenzene groups, whereas closed circles represent those containing cis-azobenzene groups (data taken from Table 2).
containing cis-azobenzene units. These orders are identical with those for increasing stability (increasing $T_m$) of the duplexes. Apparently, the formation of duplexes is also governed by the $\Delta H^o$ term.[13]

**Spectroscopic monitoring of the duplex formation by the modified oligonucleotides**

The trans-azobenzene unit in $A_iXAx_i(P)$ shows an absorption maximum at 352 nm. When $T_8$ is added to the solution (at a temperature below the $T_m$ of the trans-$A_iXAx_i(P)/T_8$ duplex), the absorption band of the azobenzene shifts towards longer wavelengths. At a temperature higher than the $T_m$ of the duplex (24.8°C), however, the spectrum is hardly changed even after addition of $T_8$. As the temperature is lowered, the concentration of the trans-$A_iXAx_i(P)/T_8$ duplex increases and accordingly the absorption maximum of the azobenzene is changed (Figure 5).

Consistently, the plot of $\lambda_{\text{max}}$ vs. temperature has a sigmoidal shape, and the midpoint is close to the $T_m$ of the duplex. Furthermore, on the formation of this duplex, a weak but explicit CD is induced in the 300 – 500 nm region (solid line in Figure 6 A). This induced CD corresponds to the absorption by the trans-azobenzene. Note that this spectrum (solid line in Figure 6 A) is obtained at 0°C, which is below the $T_m$ of the duplex (24.8°C). As expected, no CD is induced at 50°C where most of the duplex is dissociated into two single-stranded DNA oligonucleotides (dotted line in Figure 6 A). The circular dichroism in the 200–300 nm region (corresponding to the absorption of nucleic acid bases) is also strengthened.

All these results indicate that the trans-azobenzene moiety, which has a planar structure,[16] intercalates between two base pairs of the DNA duplex.[15] Consistently, the induced CD around 350 nm is very weak (Figure 6 A). This result shows that the corresponding $\pi - \pi^*$ transition moment (which is parallel to the long axis of trans-azobenzene) is parallel to the plane of DNA base pairs (as expected from the intercalation mode).[16] If the azobenzene moiety were to be placed parallel to the long axis of the duplex, a strongly positive CD should be induced.

When the trans-azobenzene moiety in $A_iXAx_i(P)$ is isomerized to the cis form by UV irradiation, a notable CD is also induced in the 200 – 500 nm region (Figure 6 B). Probably, the cis-azobenzene is not directly interacting with the DNA bases. Since the nonplanar structure of cis-azobenzene[17] is unfavorable for intercalation into DNA duplexes, it would destabilize the duplex by steric repulsion.

As shown by the thermodynamic analysis (Table 2), the cis—trans isomerization of the azobenzene moiety facilitates duplex formation because of a favorable $\Delta H^o$ term. The increased exothermicity for the trans-azobenzene-modified oligonucleotides is ascribed to the stacking interactions of the azobenzene with the adjacent base pairs. These interactions are stronger than the hydrogen bonds in Watson–Crick A/T base pairs so that the duplexes of modified oligonucleotides (containing trans-azobenzene units) are even more stable than the native duplexes (e.g., the $A_i/T_8$ duplex). When an azobenzene unit is placed in the middle of an oligonucleotide, the resulting duplexes are destabilized. This finding is consistent with the previous results that internal mismatches destabilize duplexes to a greater extent than terminal mismatches.[18]

**Conclusions**

By introducing a photoresponsive azobenzene group into oligonucleotides, the formation/dissociation of DNA duplexes has been successfully photoregulated (Figure 7). The $T_m$ values are significantly changed, irrespective of the position of the azobenzene unit in the oligonucleotides. In these photoregula-
tions, the trans-azobenzene stabilizes the duplexes by intercalation, whereas the cis-azobenzene destabilizes them. The present modified oligonucleotides are suitable for the photo-regulation of gene expression (and of various enzymatic reactions), since 1) the structural modification of the oligonucleotides is minimized, 2) the photoregulation is reversible and involves no side reaction, and 3) light is a clean and easily controllable stimulus. According to our recent experiments, these photoresponsive oligonucleotides can be successfully used as modulators of DNA elongation by T7 polymerase.

Whether DNA elongation is terminated at a desired site or proceeds down to the end of the template DNA is clearly dictated by photoinduced cis–trans isomerization of the azobenzene.

### Experimental Section

**Materials:** The phosphoramidite monomer 3 bearing an azobenzene group was synthesized according to Scheme 2 (details of the synthesis are described in the Supporting Information). 2,2-Bis(hydroxymethyl)propanoic acid, N,N’-dicyclohexylcarbodiimide, N-hydroxybenzotriazole, 4-aminooazobenzene, 4,4’-dimethoxytrityl chloride (DMT-Cl), 4-(dimethylamino)pyridine, and 1H-tetrazole (Tokyo Kasei), as well as 2-cyanoethyl N,N,N’,N’-tetraisopropylphosphoramidite (Aldrich), were commercially obtained and used without further purification. Pyridine and dimethylformamide (DMF) were distilled after being dried over CaH₂. The conventional phosphoramidite monomers, controlled-pore glass (CPG) columns, and other reagents for DNA synthesis were purchased from Glen Research Co.

**Synthesis of azobenzene-modified oligonucleotides and separation of the isomers:** All the modified oligonucleotides shown in Scheme 1 were synthesized on an automated DNA synthesizer by using the phosphoramidite monomer 3 and conventional monomers. After the recommended workup, they were purified by reversed-phase HPLC (Merck LiChrospher 100 RP-18(e) column, flow rate 0.5 mL min⁻¹, linear gradient 5→25% (25 min) acetonitrile in water containing 50 mmol dm⁻³ ammonium formate, detection at 260 nm). These modified oligonucleotides occur in two diastereomic forms (α and β, Scheme 3) due to the chirality of building block X (see Scheme 1). Furthermore, the azobenzene residue in each of them is either in the cis form or the trans form. In the present study, all of these four isomers were completely separated by the reversed-phase HPLC described above. The first and the third fractions (in the order of increasing retention time) were the cis and the trans isomers of one diastereomer, whereas the second and the fourth fractions are the cis and the trans isomers of the other diastereomer. Hereafter, the cis and the trans isomers of the former (more polar) diastereomer is designated as P, whereas the corresponding stereo-isomers of the latter (less polar) diastereomer is designated as L (e.g., trans-A₂X₃A₅(P) and cis-A₂X₃A₅(L)). With three oligonucleotides (XₐA₃X₄, XₐX₄, and GₐX₆G₈), the α and β diastereomers were not sufficiently separated by HPLC, and thus they were used as the mixtures (note that the cis and the trans isomers were completely separated even in these cases).

**Characterization of the modified oligonucleotides:** The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra (negative mode) were in satisfactory agreement (within experimental error) with the expected structures. Compounds XₐₐXₐ₃: m/z: Xₐₐ (2507); AXAₐ₃(P), 2507; AXAₐ₃(L), 2507; AₐXₐ₃(P), 2507; AₐXₐ₃(L), 2509; AₐXₐ₃(P), 2505; AₐXₐ₃(L), 2504 (calcd for [AₐXₐ₃H⁺ – H⁻]: 2504); compounds T₄XT₅ₐ: m/z: XT₅ₐ 2440; T₄XT₅ₐ(P), 2443; XT₅ₐ(L), 2441; T₄XT₅ₐ(P), 2445; T₄XT₅ₐ(L), 2446; T₄XT₅ₐ(P), 2444 (calcd for [T₄XT₅ₐH⁺ – H⁻]: 2441); GₐX₆G₈: m/z: 2616 (calcd. for [GₐX₆H⁺ – H⁻]: 2616).

**Scheme 2.** Synthesis of the phosphoramidite monomer 3. a) 4-aminooazobenzene, dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, DMF; b) 4,4’-dimethoxytrityl chloride (DMT-Cl), 4-(dimethylamino)pyridine, and 1H-tetrazole (Tokyo Kasei), as well as 2-cyanoethyl N,N,N’,N’-tetraisopropylphosphoramidite (Aldrich), were commercially obtained and used without further purification. Pyridine and dimethylformamide (DMF) were distilled after being dried over CaH₂. The conventional phosphoramidite monomers, controlled-pore glass (CPG) columns, and other reagents for DNA synthesis were purchased from Glen Research Co.

**Scheme 3.** The two diastereomers of the modified oligonucleotides (α and β) with respect to the configuration of building block X (see Scheme 1).
Photoisomerization of the azobenzene-modified oligonucleotides: In order to isomerize the azobenzene unit from the trans form to the cis form, the oligonucleotide solutions were irradiated with light from a 150-W xenon lamp through a UV-D36C filter (Asahi Technoglass Corporation). Infrared light was cut off by using a water filter. The intensity of light at the specimen was 5.3 mW cm⁻². For the cis → trans isomerization, an L-42 filter was used (the light intensity was 230 mW cm⁻²). Under these conditions, the isomerization (either cis → cis or cis → trans) was usually equilibrated within 1 min. To ensure the completion of isomerization, however, irradiation was continued for 20–30 min.

Measurement of the melting temperatures of the duplexes: The absorbance at 260 nm was monitored at pH 7.0 (10 mmol dm⁻³ phosphate buffer). The Tm value was determined from the maximum in the first derivative of the melting curve. The temperature ramp was 1.0 °C min⁻¹. The concentration of each of the DNA oligomers was 50 μmol dm⁻³, the salt concentration was 1 mol dm⁻³ NaCl (unless otherwise stated). The melting curves for cooling and heating were virtually identical to each other. In the determination of the Tm value of the cis isomer duplexes, the UV light was applied in the middle of the measurement in order to minimize the effect of thermal cis → trans isomerization. By these procedures, the fraction of the cis isomer in the specimens was kept almost constant at 70% throughout the measurement.

Spectroscopic measurements: The UV-visible spectra were measured on a JASCO model V-530 spectrophotometer, while the circular dichroism (CD) spectra were recorded on a JASCO model J-725 spectropolarimeter. Both spectrometers were equipped with programmed temperature controllers.

Determination of the thermodynamic parameters of duplex formation: The enthalpy change (ΔH°) and the entropy change (ΔS°) for the duplex formation were determined by using Equation (1).

\[ T_m = \frac{(2.303/R) \log(c/4) + (\Delta S^o/\Delta H^o)} {1} \]

where c, is the total concentration of oligonucleotides comprising both the modified oligonucleotide and its counterpart (R is the gas constant). The c values varied from 2 to 100 μmol dm⁻³. The changes in Gibbs free energy at 25 °C (ΔG°(298 K)) were calculated from the ΔH° and ΔS° values.

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[5] The number of carbon atoms between two deoxyribonucleotide residues in the main chain is three (as in natural oligonucleotides) so that the perturbation of the backbone structure of the oligonucleotides is minimized.


The photoregulation of triple-helix formation by using the isomerization of an azobenzene has also been reported: H. Asanuma, X. Liang, T. Yoshida, M. Komiyama, Angew. Chem. 2000, 112, 1372–1374; Angew. Chem. Int. Ed. 2000, 39, 1316–1318.


Note that the oligonucleotides bearing the trans-azobenzene and the ones bearing the cis-azobenzene are completely separated by the reversed-phase HPLC (see Experimental Section).

Efficient photoregulation by the trans → cis isomerization is also possible for a DNA of random sequence. For instance, 5′-CGAXGTC-3′ (more polar fraction) was hybridized with its complementary sequence (3′-GCTTCAG-5′). The Tm of the duplex in the trans form was 25 °C, whereas that of the cis form was below 5 °C (pH 7.0, [DNA] = 50 μmol dm⁻³, [NaCl] = 1 mol dm⁻³).

In the α diastereomer in Scheme 3, both the nucleic acid bases and the azobenzene residue protrude in the same direction. In water, the hydrophobic trans-azobenzene preferably stacks onto the adjacent DNA bases and is efficiently shielded from water molecules. Thus, this diastereomer is expected to be more polar than the β isomer. The P (more polar) fraction is tentatively assigned to the α isomer, whereas the L (less polar) fraction is the β isomer. The trans-azobenzene in the α isomer feasibly intercalates into the duplex. This is also consistent with the fact that the P-isomers (with trans-azobenzene) form more stable duplexes than do the L-isomers (see Table 1). In order to elucidate these configurations unambiguously, a 1H NMR study is currently under way.

[17] The planar structure is unstable, since the two ortho-hydrogen atoms cause notable steric repulsion with each other. (14)


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