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Photoregulation of DNA Triplex Formation by Azobenzene

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Abstract: Formation and dissociation of DNA triplex are reversibly photoregulated by cis ↔ trans isomerization of the azobenzene tethered to the third strand. When the azobenzene takes the trans from, a stable triplex is formed. Upon the isomerization of *trans*-azobenzene to its cis form by UV light irradiation ($300 < \lambda < 400$ nm), however, the modified oligonucleotide is removed from the target duplex. The triplex is re-formed on photoinduced cis → trans isomerization ($\lambda > 400$ nm). The photoregulating activity significantly depends on the position of azobenzene in the third strand, as well as on the geometric position (meta or para) of its amido substituent. For *m*-amidoazobenzene, the photoregulation is the most effective when it is tethered to the 5′-end of the third strand. However, *p*-amidoazobenzene should be introduced into the middle of the strand for effective regulation. In the optimal cases, the change of T_m of the triplex, caused by the cis ↔ trans isomerization of azobenzene, is greater than 30 °C. UV−visible and CD spectroscopy, as well as computer modeling studies, clearly demonstrate that the *trans*-azobenzene intercalates between the base pairs in the target duplex and thus stabilizes the triplex by stacking interactions. On the other hand, nonplanar *cis*-azobenzene destabilizes the triplex due to its steric hindrance against the adjacent base pairs.

Introduction

Triple-helix formation is one of the most promising methods for sequence-specific recognition of a DNA duplex. Various applications have already been proposed.[1] In particular, the "antigene strategy" is an elegant and versatile approach to interfere with gene expression in both transcription and replication.[2] Enormous amounts of information have been accumulated on the preparation of triple helixes which are sufficiently stable under physiological conditions and resistant against enzymatic degradation.[3] However, little has been known about the methodology to form triplexes at a given place and time and to break them at will. If triplex formation can be controlled by outer stimuli such as light and electric signal, then the scope of application should be extended.[4]

Previously, formation of a DNA duplex was photoregulated by using cis ↔ trans isomerization of azobenzene attached to one of the two strands.[5] Furthermore, in a preliminary communication,[6] triplex formation was photoregulated by tethering azobenzene to the third strand. Stable duplexes or triplexes were formed when the azobenzene took the trans form, but they were notably destabilized by *cis*-azobenzene.

In the present paper, photoregulation of triplex formation is investigated in detail. Dependencies of photoregulating activity on (1) the position and number of azobenzene in the third strand, (2) the sequence of the duplex target, and (3) the structure of the azobenzene moiety (*m*- or *p*-amidoazobenzene) are presented. Furthermore, the mechanism of photoregulation is clarified in terms of UV−visible and circular dichroism (CD) spectroscopy as well as thermodynamic and computer modeling analyses.

Results

Photoregulation of DNA Triplex Formation by *m*-Amidoazobenzene-Tethered Oligonucleotides. The X^m involving *meta*-amidoazobenzene (−(C═O)−NH−azobenzene) is introduced to various positions in homopyrimidine sequences (see Scheme 1). Typical melting curves of X^mT_{13}/a/t triplex are presented in Figure 1. The third strand X^mT_{13}, in which *m*-amidoazobenzene is tethered to the 5′-end of a 13-mer homothymidine, binds to the A_{13}/T_{13} portion in the a/t duplex (Scheme 1) to form a triplex. Before UV light irradiation, the T_m is 29.6 °C (the solid line in Figure 1). Here, the azobenzene

(1) (a) Crooke, S. T. *Methods Enzymol.* **2000**, *313*, 3−45. (b) Hélène, C.; Thuong, N. T.; Harel-Bellan, A. *Ann. N.Y. Acad. Sci.* **1992**, *660*, 27−33. (c) Xu, X. S.; Glazer, P. M.; Wang, G. *Gene* **2000**, *242*, 219−228. (d) Chan, P. P.; Glazer, P. M. *J. Mol. Med.* **1997**, *242*, 267−282.

(2) (a) Wang, G.; Seidman, M. M.; Glazer, P. M. *Science* **1996**, *271*, 802−805. (b) Barre, F. X.; Giovannangeli, C.; Hélène, C.; Harel-Bellan, A. *Nucleic Acids Res.* **1999**, *27*, 743−749. (c) Cogoi, S.; Papozzi, V.; Quadrifoglio, F.; Xodo, L. *Biochemistry* **2001**, *40*, 1135−1143.

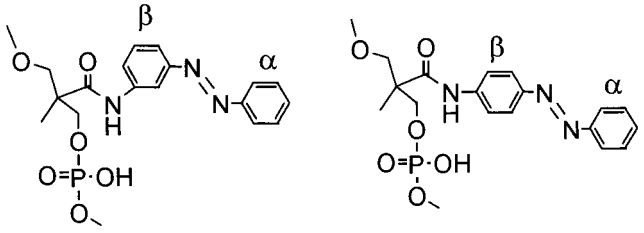
(3) (a) Silver, G. C.; Sun, J. S.; Nguyen, C. H.; Boutorine, A. S.; Bisagni, E.; Hélène, C. *J. Am. Chem. Soc.* **1997**, *119*, 263−268. (b) Maruyama, A.; Katoh, M.; Ishihara, T.; Akaike, T. *Bioconjugate Chem.* **1997**, *8*, 3−6. (c) Thomas, T.; Thomas, T. J. *Biochemistry* **1993**, *32*, 14068−14074.

(4) Photoresponsive oligonucleotides to regulate DNA functions were synthesized: (a) Fujimoto, K.; Matsuda, S.; Takahashi, N.; Saito, I. *J. Am. Chem. Soc.* **2000**, *122*, 5646−5647. (b) Grigoriev, M.; Praseuth, D.; Guieysse, A. L.; Robin, P.; Thuong, N. T.; Hélène, C.; Harel-Bellan, A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3501−3505. However, few reports have been made on the photoregulation of triplex formation.

(5) (a) Asanuma, H.; Ito, T.; Yoshida, T.; Liang X. G.; Komiyama, M. *Angew. Chem., Int. Ed.* **1999**, *38*, 2393−2395. (b) Asanuma, H.; Liang X. G.; Yoshida, T.; Komiyama, M. *ChemBioChem.* **2000**, *2*, 39−44. (c) Asanuma, H.; Liang, X. G.; Komiyama, M. *Tetrahedron Lett.* **2000**, *41(7)*, 1055−1058. (d) Yamazawa, A.; Liang, X. G.; Asanuma, H.; Komiyama, M. *Angew Chem., Int. Ed.* **2000**, *39*, 2356−2358.

(6) Asanuma, H.; Liang, X. G.; Yamazawa, A.; Yoshida T.; Komiyama, M. *Angew. Chem., Int. Ed.* **2000**, *39*, 1316−1318.

Scheme 1. Structures of the Azobenzene Units (X^m, X^p) as Well as the Sequences of the Duplex Targets and the Third Strands Used in This Study[a]



X^m X^p

Duplex targets

a/t

5′-CGTCGGTTT-AAAAAAAAAAAAA-TTTCGTGGC-3′
3′-GCAGCCAAA-TTTTTTTTTTTTT-AAAGCACCG-5′

ag/ct

5'-CGAGTT-AAGAAGAAAAAAGA-TTGAGC-3'
3'-GCTCAA-TTCTTCTTTTTTCT-AACTCG-5'

The third strands:

X^mT_{13}: 5′-X^mTTTTTTTTTTTTT-3′
TX^mT_{12}: 5′-TX^mTTTTTTTTTTTT-3′
$T_2X^mT_{11}$: 5′-TTX^mTTTTTTTTTTT-3′
$T_6X^mT_7$: 5′-TTTTTTX^mTTTTTTT-3′
$T_{13}X^m$: 5′-TTTTTTTTTTTTTX^m-3′
$X^mT_{11}C_3$: 5′-X^mTTCTTCTTTTTTCT-3′

T_{13}: 5′-TTTTTTTTTTTTT-3′
X^pT_{13}: 5′-X^pTTTTTTTTTTTTT-3′
TX^pT_{12}: 5′-TX^pTTTTTTTTTTTT-3′
$T_2X^pT_{11}$: 5′-TTX^pTTTTTTTTTTT-3′
$T_3X^pT_{10}$: 5′-TTTX^pTTTTTTTTTT-3′
$T_4X^pT_9$: 5′-TTTTX^pTTTTTTTTT-3′
$T_5X^pT_8$: 5′-TTTTTX^pTTTTTTTT-3′
$T_6X^pT_7$: 5′-TTTTTTX^pTTTTTTT-3′
$T_7X^pT_6$: 5′-TTTTTTTX^pTTTTTT-3′
$T_{13}X^p$: 5′-TTTTTTTTTTTTTX^p-3′
$X^pT_6X^pT_7$: 5′-X^pTTTTTTX^pTTTTTTT-3′

[a] Two benzene rings on azobenzene are designated as α and β.

mostly (>90%) takes the trans form, as indicated by the UV−visible spectrum and the reversed-phase HPLC. Upon irradiating the solution with UV light ($300 < \lambda < 400$ nm) at 50 °C, the azobenzene in X^mT_{13} is isomerized to its cis form (> 80%). Concurrently, the T_m is lowered to 10.4 °C (the dotted line). The difference in T_m between the trans form and the cis form (ΔT_m) is as large as 19.2 °C. By irradiation with visible light ($\lambda > 400$ nm), the *cis*-azobenzene is isomerized (about 90%) back to the trans form, and the melting curve is almost superimposed with that (the solid line) before the UV light irradiation. This reversible change is repeated without apparent deterioration.

The T_m values for the triplexes of a series of modified homothymidines ($T_iX^mT_j$) with the target a/t duplex are presented in Table 1. Two diastereomers of $T_iX^mT_j$, with respect to the chirality of X^m, were completely separated by the HPLC, except for X^mT_{13} and $T_{13}X^m$ (see Experimental Section). The T_m values for each of the diastereomers (polar fractions and less polar fractions) are listed in this table. The differences between these diastereomers are rather small, although the effects were far more remarkable in the photoregulation of duplex formation.[5b]

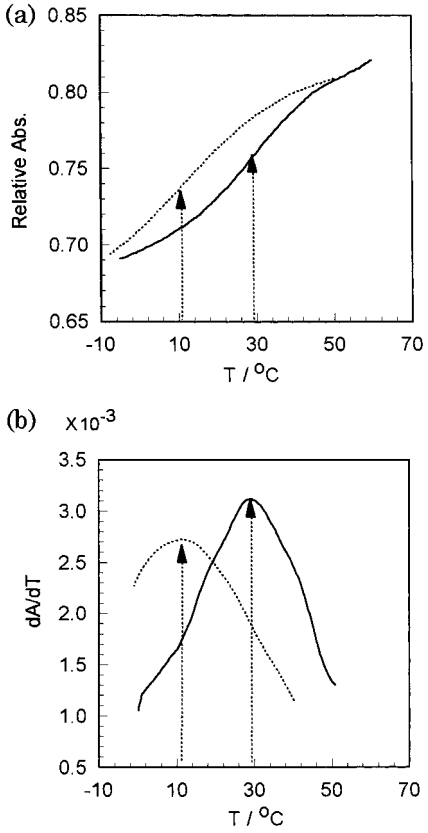


Figure 1. Melting curves (a) and their first derivatives (b) for the *trans*-X^mT_{13}/a/t triplex (solid line) and the *cis*-X^mT_{13}/a/t triplex (dotted line). $[X^mT_{13}] = [a/t] = 2.0$ μM, $[MgCl_2] = 0.2$ M, 10 mM HEPES buffer, pH 7.0. The melting curve of duplex target a/t ($T_m = 73.0$ °C) is not shown here.

Table 1. Melting Temperatures (T_m's) of Triplexes Formed from the Duplex Target a/t and the Third Strand Containing *m*-amidoazobenzene at Various Positions[a]

	polar fraction[b]			less polar fraction[b]		
	T_m/°C			T_m/°C		
third strand	trans	cis	ΔT_m/°C[c]	trans	cis	ΔT_m/°C
T_{13}	40.0					
X^mT_{13}[d]	46.3	26.2	20.1			
TX^mT_{12}	31.2	27.8	3.4	32.5	27.0	5.5
$T_2X^mT_{11}$	22.0	18.9	3.1	22.6	20.0	2.6
$T_6X^mT_7$	24.8	20.3	4.5	29.5	16.2	13.3
$T_{13}X^m$ [d]	39.3	37.2	2.1			

[a] $[T_iX^mT_j] = [a/t] = 2$ μM, $[MgCl_2] = 1.0$ M, and pH 7.0 (10 mM HEPES buffer). [b] Two diastereomers with respect to the chirality of X^m. The isomer which elutes faster in the reversed-phase HPLC is designated as the polar fraction (see Experimental Section for details). [c] Change of T_m induced by the trans ↔ cis isomerization. [d] The diastereomers could not be separated by HPLC.

The triplex is most stable when *trans*-azobenzene is tethered to the 5′-end of the third strand (X^mT_{13}). Its T_m is even higher (by 6.3 °C) than that of the native triplex T_{13}/a/t.[7] With the *trans*-X^m inside the strand, the triplex is less stable (the T_m is 15−22 °C lower than that of X^mT_{13}). On the other hand, the stability of the triplex of *cis*-azobenzene is rather independent of the position of X^m. As a result, the ΔT_m for X^mT_{13} (20.1 °C) is far greater than the values (3−5 °C) for TX^mT_{12}, $T_2X^mT_{11}$, and $T_6X^mT_7$. When the azobenzene is at the 3′-end ($T_{13}X^m$),

(7) The increase in T_m promotes the formation of triplexes under physiological conditions.

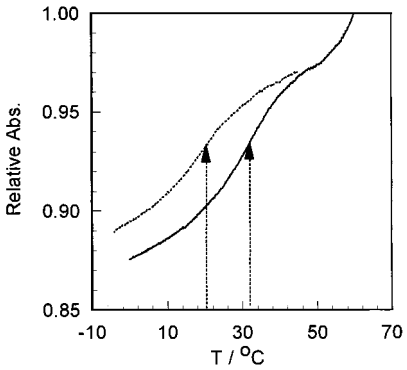


Figure 2. Melting curves for the triplex *trans*-$X^mT_{11}C_3$/ag/ct (solid line) and *cis*-$X^mT_{11}C_3$/ag/ct (dotted line). [$X^mT_{11}C_3$] = 1.5 μM, [ag/ct] = 1.0 μM, [KCl] = 140 mM, [$MgCl_2$] = 1.5 mM, [spermine] = 0.8 mM, 15 mM sodium cacodylate buffer, pH 6.9.

Table 2. Melting Temperatures (T_m's) of Triplexes Formed from the Duplex Target a/t and the Third Strand Containing *p*-amidoazobenzene at Various Positions[a]

	polar fraction[b]			less polar fraction[b]		
	T_m/°C			T_m/°C		
third strand	trans	cis	ΔT_m/°C[c]	trans	cis	ΔT_m/°C
T_{13}	40.0					
X^pT_{13}[d]	47.3	33.0	14.3			
TX^pT_{12}	32.9	24.1	8.8	36.1	23.8	12.3
$T_2X^pT_{11}$	33.9	14.7	19.2	35.0	18.5	16.5
$T_3X^pT_{10}$	36.7	10.8	25.9	36.6	10.2	26.4
$T_4X^pT_9$	41.0	6.1	34.9	40.8	7.2	33.6
$T_5X^pT_8$	41.7	4.3	37.4	41.2	5.7	34.5
$T_6X^pT_7$	42.1	7.0	35.1	43.3	9.0	34.3
$T_7X^pT_6$	42.2	6.0	36.2	42.5	8.0	34.5
$T_{13}X^p$[d]	40.6	34.5	6.1			
$X^pT_6X^pT_7$	48.4	<0	>48.4	46.7	<0	>46.7

[a] [$T_iX^pT_j$] = [a/t] = 2 μM, [$MgCl_2$] = 1.0 M, and pH 7.0 (10 mM HEPES buffer). [b] Two diastereomers with respect to the chirality of X^p. [c] Change of T_m induced by the trans ↔ cis isomerization. [d] The diastereomers could not be separated by HPLC.

the triplex is stable with either *trans*-azobenzene or its cis isomer, and thus ΔT_m is small.

Formation of triplexes containing a C^+GC triplet [8] (e.g., the $X^mT_{11}C_3$/ag/ct triplex in Scheme 1) is also successfully photoregulated, as shown in Figure 2. The ΔT_m between the trans and the cis forms is 11.4 °C. When one of the A/T pairs at either the 5′-end or the 3′-end of the A_{13}/T_{13} portion in an a/t duplex is changed to T/A, G/C, or C/G, the change in ΔT_m for the triplexes with X^mT_{13} is marginal (ΔT_m = 16.6−22.5 °C, see Supporting Information Table 1). The present photoregulation is applicable to versatile combinations of oligonucleotides.

Photoregulation of DNA Triplex Formation by *p*-Amidoazobenzene-Tethered Oligonucleotides. The T_m values for the modified homothymidines bearing *p*-amidoazobenzene (X^p in Scheme 1) are shown in Table 2. When the *p*-amidoazobenzene is at the 5′-end (X^pT_{13}), the triplex of the trans form is most stable, but the cis triplex is also rather stable. The ΔT_m is 14.3 °C. The ΔT_m of TX^pT_{12}/a/t is slightly smaller than that of X^pT_{13}/a/t, mainly because the trans triplex is less stable. It is noteworthy that the photoregulation is especially efficient when X^p is tethered to the middle of the third strand. The *cis*-azobenzene in $T_4X^pT_9$, $T_5X^pT_8$, $T_6X^pT_7$, and $T_7X^pT_6$ enormously

(8) The cytosines of the third strand are protonated even at neutral pH: Leitner, D.; Schröder, W.; Weisz, K. *Biochemistry* **2000**, *39*, 5886−5892.

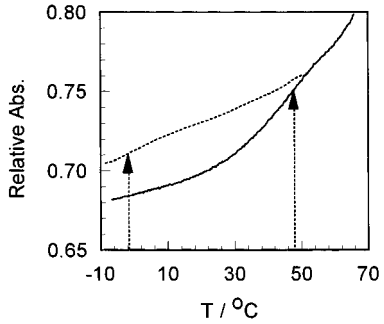


Figure 3. Enhanced change of melting profiles of triplex formation between $X^pT_6X^pT_7$ (polar fraction, involving two azobenzene moieties) and a/t duplex either in the trans (solid line) or the cis form (dotted line). T_m values are listed in the last line of Table 2 with those of the less polar fraction.

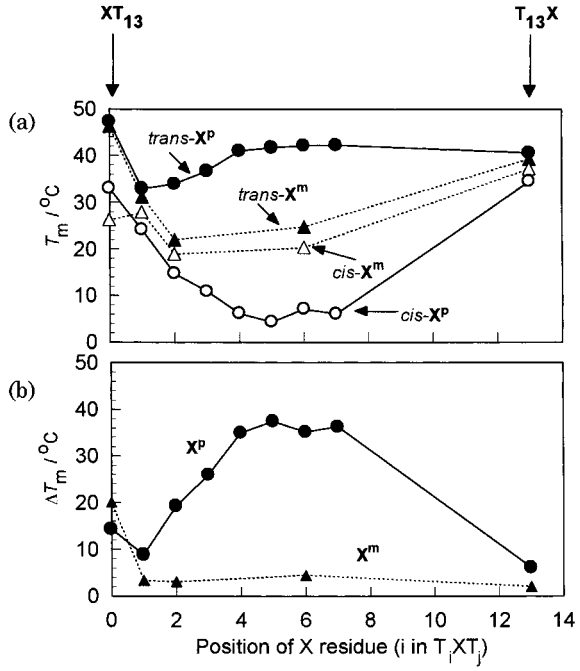


Figure 4. Effect of the position of the X residue in homothymidine on the T_m of the triplex (a). Closed circles, trans form of *p*-amidoazobenzene; open circles, cis form of *p*-amidoazobenzene; closed triangles, trans form of *m*-amidoazobenzene; open triangles, cis form of *m*-amidoazobenzene. The data for the polar fractions in Table 1 and 2 are plotted. In (b), the ΔT_m values induced by the cis → trans isomerization are shown for *p*-amidoazobenzene (closed circles) and *m*-amidoazobenzene (closed triangles). The horizontal axis refers to the *i* value in $T_iX^pT_j$ or $T_iX^mT_j$.

destabilizes the triplex (T_m = 4−7 °C), and thus ΔT_m's for these modified oligonucleotides exceed 30 °C. For all the sequences, the differences between the diastereomers are marginal.

Still greater ΔT_m (>48 °C) is obtained by introducing two *p*-amidoazobenzene molecules to one homothymidine sequence (Figure 3). The T_m of the *trans*-$X^pT_6X^pT_7$/a/t triplex is 48.4 °C, whereas the corresponding value of *cis*-$X^pT_6X^pT_7$/a/t is too low to be measured precisely (<0 °C).[9]

Comparison of the Photoregulating Activity between *m*- and *p*-Amidoazobenzene-Modified Oligonucleotides. The melting temperatures of $T_iX^mT_j$/a/t and $T_iX^pT_j$/a/t are graphically summarized in Figure 4 as a function of the position of the X residue in homothymidine. When the X residue is tethered to

(9) Before UV irradiation, more than 85% of the azobenzene moieties in $X^pT_6X^pT_7$ adopt the trans form. Upon UV light irradiation at 50 °C, both of the *trans*-azobenzenes are isomerized to the cis form (cis fraction is about 70%).

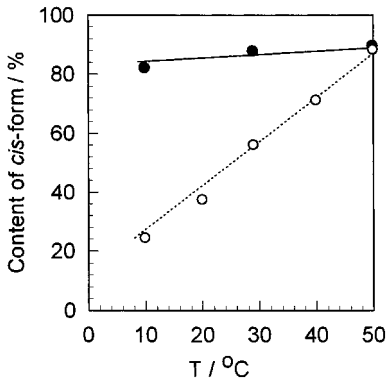


Figure 5. Photoisomerization of the azobenzene moiety from the trans to the cis form by irradiating UV light to X^mT_{13} in the absence (closed circles) or the presence (open circles) of the a/t duplex target at various temperatures. $[X^mT_{13}] = [a/t] = 10\ \mu M$, $[MgCl_2] = 0.2$ M, 10 mM HEPES buffer, pH 7.0. T_m's of *trans*- and *cis*-X^mT_{13}/a/t are 37.9 and 20.6 °C, respectively.

the end of the strand (XT_{13} or $T_{13}X$), the geometric position of the amido group on the azobenzene has only a small effect on the T_m. In the trans forms, both meta and para isomers stabilize the triplexes in similar magnitudes. Destabilization of triplexes by their cis forms is also similar. Accordingly, the ΔT_m's induced by the cis → trans isomerization are close to each other.

On the other hand, *m*-amidoazobenzene inside the third strand significantly lowers the T_m, and the magnitudes of change by the trans form are almost the same as those by the cis form (compare the open triangles with the closed triangles in Figure 4a). Thus, the ΔT_m is small (see the triangles in Figure 4b). The ΔT_m is maximized with X^mT_{13}. This trend is entirely inverted with *p*-amidoazobenzene. When X^p is tethered inside the strand, the T_m of the cis form is greatly lowered (the open circles in Figure 4a), but the T_m of the trans form is less affected (the closed circles). Thus, $T_5X^pT_8$, $T_6X^pT_7$, and $T_7X^pT_6$ provide the maximum ΔT_m (>30 °C).

Efficiency of Photoinduced Trans → Cis Isomerization of Modified Oligonucleotides at Various Temperatures. For single-stranded X^mT_{13}, the efficiency of trans → cis isomerization by UV light irradiation is not much dependent on the temperature (the closed circles in Figure 5). The photoequilibrium is attained within 1 min. When the a/t duplex is added to the solution, however, the temperature dependency is now remarkable (the open circles). The trans → cis isomerization is less efficient at lower temperatures, where the triplex is firmly formed. The amount of *cis*-azobenzene in photoequilibrium is only 24% for the photoirradiation at 10 °C. The isomerization efficiency rapidly increases with increasing temperature. Note that, in the present paper, the melting temperatures of *cis*-azobenzene-bearing strands are measured after the UV irradiation at 50 °C. There, the photoisomerization occurs efficiently.[10]

Thermodynamic and Spectroscopic Analysis of the Photoregulation. To shed light on the mechanism of photoregulation, thermodynamic and spectroscopic analyses were carried out. The exothermic change ($-\Delta H°$) for *trans*-X^mT_{13}/a/t is about 40 kJ mol^{-1} greater than that for *cis*-X^mT_{13}/a/t (see Table 3). The entropy term partially compensates this factor. These facts indicate that favorable $\Delta H°$ is the primary driving force for the efficient stabilization of triplex by the *trans*-azobenzene. For other triplexes containing azobenzene, similar results were obtained (data not shown).

(10) The dissociation of triplex by UV irradiation has been experimentally evidenced: Supporting Information Figure 1.

Table 3. Thermodynamic Parameters for the Triplex Formation between T_{13} or X^mT_{13} and the a/t Duplex[a]

third strand	T_m/°C[b]	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J K^{-1} mol^{-1})	$\Delta G°$(310 K) (kJ mol^{-1})
T_{13}	22.5	−176	−481	−27.0
trans-X^mT_{13}	30.2	−159	−409	−31.9
cis-X^mT_{13}	10.7	−119	−304	−24.7

[a] $[MgCl_2] = 0.2$ M, at pH 7.0 (10 mM HEPES buffer). [b] $[T_{13}] = [X^mT_{13}] = [a/t] = 2\ \mu M$.

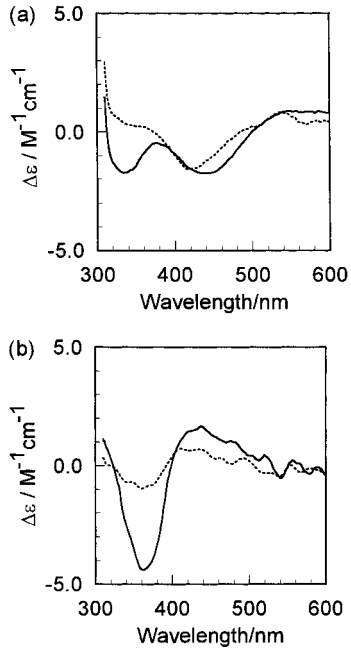


Figure 6. Circular dichroism spectra of X^mT_{13}/a/t (a) and X^pT_{13}/a/t (b) at 0 °C, either in the trans (solid line) or cis form (dotted line). $[X^mT_{13}] = [X^pT_{13}] = [a/t] = 10\ \mu M$, $[MgCl_2] = 0.2$ M, 10 mM HEPES buffer, pH 7.0.

At 0 °C, the *trans*-azobenzene in single-stranded *trans*-X^mT_{13} exhibits an absorption maximum at 325 nm which corresponds to the $\pi-\pi^*$ transition. When the a/t duplex is added and the triplex is formed, the absorption maximum shifts toward longer wavelength (331 nm; see Supporting Information Figures 2 and 3). This bathochromic shift is consistent with the intercalation of *trans*-azobenzene into the base pairs in the triplex.[11] As expected, the change is almost nil when the temperatures are higher than the T_m of triplex. With the *cis*-azobenzene, however, these spectral changes are small. On the formation of X^mT_{13}/a/t and X^pT_{13}/a/t triplexes, weak but explicit CD is induced in the 300−500 nm region (Figure 6). At temperatures above the T_m, almost no CD is induced.

Molecular Modeling of the Triplexes Bearing Azobenzene. The structures of X^mT_{13}/a/t, X^pT_{13}/a/t, $T_6X^mT_7$/a/t, and $T_6X^pT_7$/a/t, determined by molecular modeling, are presented in Figures 7 and 8.[11] Only the azobenzene and its neighboring base pairs

(11) Both diastereomers with respect to the chirality of the X residue were subjected to molecular modeling. Since no significant difference was observed between the energy-minimized structures for these two diastereomers, only one of them is presented: in Figure 7, the diastereomer taking the *R*-configuration of the X residue is depicted, while that of *S*-configuration is presented in Figure 8. Note that the azobenzene moieties in these figures protrude in the same direction with respect to the backbone, although the expression of the absolute configuration (*R* or *S*) is reversed.

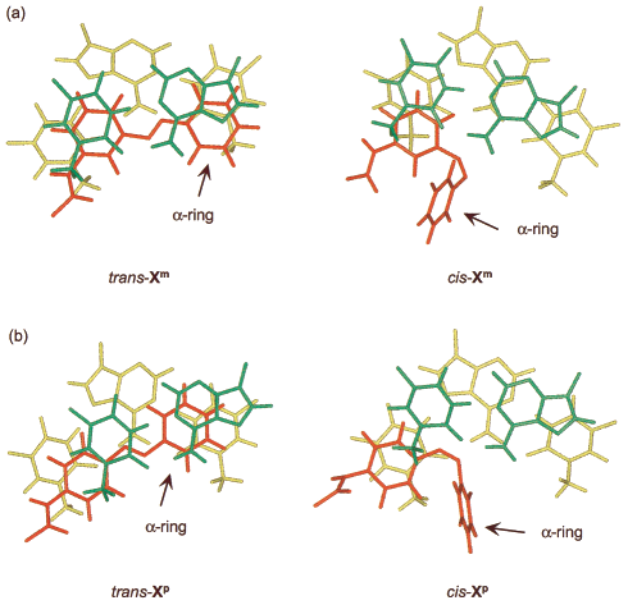


Figure 7. Energy-minimized structures of X^mT_{13}/a/t (a) and X^pT_{13}/a/t (b) either in the trans (left) or the cis form (right) obtained from computer modeling, viewed along the helix axis. The azobenzene moiety (red) and its neighboring base pairs are displayed for clarity.

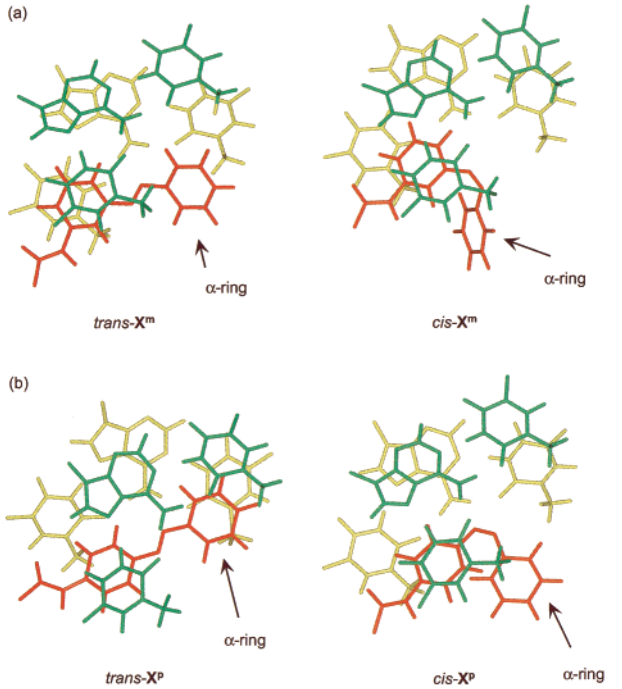


Figure 8. Energy-minimized structures of $T_6X^mT_7$/a/t (a) and $T_6X^pT_7$/a/t (b) either in the trans (left) or the cis form (right) obtained from computer modeling, viewed along the helix axis. The zobenzene moiety (red) and its neighboring base pairs are displayed for clarity.

are shown for the purpose of clarity. The *trans*-azobenzene in X^mT_{13}/a/t and X^pT_{13}/a/t intercalates between the adjacent base pairs in almost the same manner (the left-hand side in Figure 7): both α and *β* rings of azobenzene are intercalated between the base pairs. In *cis*-X^mT_{13}/a/t and *cis*-X^pT_{13}/a/t, however, the α ring flips out from the base pairs (the right-hand side).

When the azobenzene is located in the middle of the third strand, the manners of intercalation of the *trans*-azobenzene in the triplexes are significantly dependent on the position of its substituent. The α ring of *m*-amidoazobenzene in *trans*-$T_6X^mT_7$/a/t is located in the wide-spaced major groove (Figure 8a, left). Direct interaction with the thymine or the adenine of the a/t duplex is unlikely. On the other hand, the α ring of *p*-amidoazobenzene in *trans*-$T_6X^pT_7$/a/t is located just near the thymine of the a/t duplex (Figure 8b, left). When the azobenzene takes the cis form, the α ring flips out from the base pairs for both *p*- and *m*-amidoazobenzene (Figure 8a,b, right).

Discussion

Mechanism of Photoregulation Induced by Cis ↔ Trans Isomerization of Azobenzene. All the results demonstrate that triplexes are stabilized more greatly by *trans*-azobenzene than by *cis*-azobenzene. The magnitude of stabilization (or destabilization) depends on the sequence and geometry of the amido group on the azobenzene (Tables 1 and 2). Thus, visible light irradiation (cis → trans isomerization of azobenzene) induces the formation of triplexes, whereas UV light irradiation (trans → cis isomerization) induces its dissociation. The planar *trans*-azobenzene intercalates between the adjacent base pairs, as clearly evidenced by the spectroscopy (bathochromic shift of the absorption maximum[12] and negatively induced CD in Figure 6).[13] This structure is also supported by the computer modeling (Figures 7 and 8). Both α and *β* rings of azobenzene moiety are completely intercalated.

Unlike *trans*-azobenzene, the photoisomerized cis form takes the nonplanar structure due to the steric repulsion between ortho hydrogens.[14] Nonplanar *cis*-azobenzene is unfavorable for the intercalation or, rather, seriously destabilizes the triplex due to steric hindrance against the adjacent base pairs. These arguments are also consistent with the computer modeling in Figures 7 and 8. The smaller induced CD of the *cis*-X^mT_{13} than that of the *trans*-X^mT_{13} also supports the incomplete intercalation of *cis*-azobenzene.

On the basis of these results, the mechanism of the present photoregulation of triplex formation can be proposed as follows: the incorporated *trans*-azobenzene intercalates between base pairs of the DNA duplex target and stabilizes the triplex by the stacking interaction, while nonplanar *cis*-azobenzene destabilizes it due to steric repulsion.[15] The greater exothermicity ($-\Delta H°$) for the formation of *trans*-X^mT_{13}/a/t than the corresponding value for the cis isomer is mostly ascribed to both the free energy of stacking interaction and the absence of steric repulsion.

Difference of the Photoregulating Activity between *m*- and *p*-Amidoazobenzene. When an azobenzene is tethered to the 5′-end of homothymidine, the geometric position of the amido group on the azobenzene does not significantly affect the T_m of the triplex in either the trans form or the cis form (Figure 4). Since the X residue at the 5′-end has a sufficient freedom of molecular movement, the trans forms of both *m*- and *p*-

(12) A similar bathochromic shift was observed on the triplex formation of *p*-amidoazobenzene-attached oligonucleotides. See ref 6.

(13) (a) Kubista, M.; Åkerman, B.; Nordén, B. *J. Phys. Chem.* **1988**, *92*, 2352−2356. (b) Lyng, R.; Rodger, A.; Nordén, B. *Biopolymers* **1992**, *32*, 1201−1214. (c) Pradhan, P.; Jernström, B.; Seidel, A.; Nordén, B.; Gräslund, A. *Biochemistry* **1998**, *37*, 4664−4673.

(14) Robertson, J. M. *J. Chem. Soc.* **1939**, 232−236.

(15) Consistently, a phenylazonaphthalene, introduced into the 5′-end of T_{13}, showed a greater photoregulating activity on triplex formation than did an azobenzene. Its larger size is more favorable for both the stacking interactions (trans form) and the steric repulsion (cis form): Liang, X. G.; Asanuma, H.; Komiyama, M. *Tetrahedron Lett.* **2001**, *42*(*38*), 6723−6725.

amidoazobenzene can take the position which is the most appropriate for the intercalation. Consistently, X^mT_{13}/a/t and X^pT_{13}/a/t have almost the same energy-minimized structures (Figure 7). Similarly, the steric hindrance caused by the *cis*-azobenzene can be minimized due to its notable mobility. As a result, the T_m for X^m is similar to that for X^p in both their trans and cis forms.

In contrast, a significant difference between X^m and X^p is brought about when they are incorporated to the interior of the third strand (see Figure 4). As X^m (in either the trans form or the cis form) is shifted from the 5′-end of homothymidine to its center, the T_m's of the corresponding triplexes are gradually lowered. The molecular movement of *m*-amidoazobenzene is considerably restricted here so that its overlapping with the base pairs of the duplex cannot be optimized (Figure 8a). Thus, the triplex is not much stabilized. The steric repulsion in the cis isomer is not drastic, since its α ring is located in the wide-spaced major groove. Accordingly, the trans → cis isomerization induces only small change of T_m.

For the para isomer X^p in the middle of the third strand, the trans triplex is notably stabilized, and the T_m is not much dependent on the position of X^p. The *trans-p*-amidoazobenzene protrudes in the direction of thymine in the duplex target and intercalates there (Figure 8b). The triplex is sufficiently stabilized through stacking interactions.[16] In contrast, the T_m for the cis triplex is greatly dependent on the position of X^p and takes a steep minimum when the X^p residue is placed in the center. The nonplanar *cis*-azobenzene cannot completely intercalate due to the steric hindrance between the α ring and the thymine in the duplex. Rather, *cis*-azobenzene disturbs the structure of the triplex drastically, and the α ring is forced to come out to the groove (Figure 8b). Consequently, the cis form enormously destabilizes the triplex, and thus the T_m of the triplex involving *cis*-X^p is far lower than that of *cis*-X^m.

Conclusion

(1) DNA triplex formation is reversibly photoregulated by introducing azobenzene into the side chain of the third strand. This photoregulation is effective for all the duplex targets investigated in this study. When two *p*-amidoazobenzene residues are simultaneously introduced into the third strand, the photoregulation is still more effective (ΔT_m > 48 °C).

(2) Stabilization of the triplex by the *trans*-azobenzene is attributed to the intercalation of the planar trans form between base pairs of the DNA duplex, while the triplex is destabilized by nonplanar *cis*-azobenzene due to steric hindrance. This is the origin of the present photoregulation.

(3) For the *m*-amidoazobenzene-modified third strand, photoregulation is the most effective when the azobenzene is tethered to the 5′-end (ΔT_m = 19.2 °C). For *p*-amidoazobenzene, however, the ΔT_m is maximized (>30 °C) by attaching it to the interior position.

Application of this method to the photoregulation of various enzymatic reactions (based on the antigene strategy) is currently underway.

Experimental Section

Materials. The phosphoramidite monomers carrying an azobenzene were synthesized as described previously.[5a] All the conventional phosphoramidite monomers, CPG columns, reagents for DNA synthesis, and Poly-Pak cartridges were purchased from Glen Research Co. Alkaline phosphatase was purchased from Wako Pure Chemical Industries Ltd., and snake venom phosphodiesterase was from Boehringer Mannheim.

Synthesis and Purification of the Oligonucleotides. The oligonucleotides used in this study were synthesized on an ABI DNA/RNA synthesizer model 394 by phosphoramidite chemistry. The synthesis was carried out according to the conventional protocols, and no special program was needed. The coupling efficiency of the amidite monomer carrying an azobenzene was as high as those of conventional monomers, as judged from the coloration of the released trityl cation. The oligomers involving azobenzene moieties were successfully deprotected by treating with 25% ammonia solution for 12 h at 50 °C without any side reactions.

To introduce an azobenzene to the 3′-end, 5′-CE (β-cyanoethyl phosphoramidites) and the 5′-supports (CPG columns) were used, and the synthesis was achieved from the 5′ to 3′ terminus. The purification was done by using Poly-Pak cartridges and then by reversed-phase HPLC (Merck LiChrospher 100 RP-18(e) column). The eluent was a linear gradient of the mixture of acetonitrile and H_2O (containing 50 mM ammonium formate): from 12/88 to 15/85 in 60 min. All the oligonucleotides used were characterized by MALDI-TOFMS (negative mode), where the differences between calculated and observed values were within ±0.1% (see Supporting Information Table 2).

Each modified oligonucleotide carrying one azobenzene moiety has two diastereomers with respect to the chirality of the carbon atom in X^m and X^p. Moreover, the azobenzene moiety in each diastereomer involves trans and cis isomers. In the present study, these four isomers were completely separated by reversed-phase HPLC under the conditions 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 (designated as "polar fraction"), and the second and the fourth fractions were the cis and trans isomers of the other diastereomer ("less polar fraction").[5a] When an azobenzene was introduced to the 5′- or 3′-end of an oligonucleotide (X^mT_{13}, X^pT_{13}, $T_{13}X^m$, and $T_{13}X^p$), the diastereomers could not be sufficiently separated by HPLC, and thus they were used as the mixtures. In the case of $X^pT_6X^pT_7$, only two diastereomers of the four possible ones, probably based on the chirality of the inside X^p, could be separated.

The concentration of oligonucleotides was determined by enzymatic digestion with alkaline phosphatase and snake venom phosphodiesterase. Experimental error was within 10%. The concentration was given on a per-strand basis.

Photoisomerization of the Azobenzene Tethered to Oligonucleotides. The light source for the photoirradiation was a 150 W xenon lamp. For the trans → cis isomerization, an UV-D36C filter (Asahi Tech. Co.) was used, and the UV light (300 < λ < 400 nm; 5.3 mW cm^{-2}) was irradiated at 50 °C (unless noted otherwise). The cis → trans isomerization was achieved by irradiating the visible light (λ > 400 nm; 230 mW cm^{-2}) obtained by using an L-42 filter (Asahi Tech. Co.). In both cases, a water filter was used to cut off infrared light. The irradiation time was 10 min. The isomerization was monitored by UV−visible spectra and/or HPLC analysis. Before HPLC analysis, the triplex was diluted to 1/100 by water, and the third strand was dissociated from the duplex target. By these treatments, the fraction of the cis isomer of *p*-amidoazobenzene was kept above 70% throughout the measurement. The cis isomer of *m*-amidoazobenzene was much more stable, so its content was kept >80%.[17]

Measurement of Melting Temperature. The melting curve of the triplex was obtained by measuring the change of absorbance at 280 nm versus temperature. A Jasco model V-530 spectrophotometer

(16) The much higher T_m of *trans*-X^p than that of *trans*-X^m is ascribed to the more efficient intercalation of the α ring.

(17) The cis → trans thermal isomerization of *m*-amidoazobenzene is quite slow (ref 5c). At 37 °C, the half-life for the cis form is 64 h, while the corresponding value for the para isomer is 1 h. This feature is advantageous for practical applications.

equipped with a programmable temperature controller was used. The melting temperature (T_m) was determined from the maximum in the first derivative of the melting curve. Both of the heating and cooling curves were measured, and the T_m values obtained from them coincided with each other within 2.0 °C. The T_m values presented here are an average of 2−4 independent experiments. The error of T_m values is ±1.0 °C.

The mole ratio of the center strand (containing a homopurine portion), the second strand (containing a homopyrimidine portion), and the third strand was 1:1.2:1. When the third strand was composed of T only (a/t duplex target), buffer containing 0.2 or 1.0 M $MgCl_2$ and 10 mM HEPES (pH 7.0) was used, and the temperature ramp for the T_m measurement was 1.0 °C min^{-1}. When the third strand was composed of both T and C (ag/ct duplex target), the buffer containing 140 mM KCl, 15 mM sodium cacodylate (pH 6.9), 1.5 mM $MgCl_2$, and 0.8 mM spermine was used. In this case, the temperature ramp was as small as 0.1 °C min^{-1} since hybridization occurred so slowly under the conditions employed.[18]

Spectroscopic Measurements. The UV−visible spectra and CD spectra were measured on a Jasco model V-530 and a Jasco model J-725, respectively. Both of them were equipped with programmed temperature controllers.

Determination of Thermodynamic Parameters for the DNA Triplex Formation. The enthalpy change ($\Delta H°$) and the entropy change ($\Delta S°$) were determined according to eq 1.[19]

$$T_m^{-1} = (2.30R/\Delta H°)\log(C_t/4) + (\Delta S°/\Delta H°) \quad (1)$$

where C_t is the sum of the concentration of the duplex target and that of the third strand (R is the gas constant). The C_t values were changed from 1.0 to 50 μM. The change of Gibbs free energy at 37 °C ($\Delta G°$(310)) was calculated from the $\Delta H°$ and the $\Delta S°$.

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Molecular Modeling. The Insight II/Discover 98.0 program package was used for molecular modeling by conformational energy minimization. The azobenzene residue attached to the third strand was built using the graphical program. The phosphate group was treated as a nondissociating state. Neither water nor positively charged counterions were explicitly included in the energy minimization. Their effects were simulated by a sigmoidal, distance-dependent, dielectric function. B-type duplex target a/t (Scheme 1) was assumed, and the CFF force field was used for calculation. Computations were carried out on a Silicon Graphics Octane workstation with the operating system IRIX64 Release 6.5.

As an initial structure of calculation, an azobenzene moiety was placed between the base pairs since spectroscopic analyses strongly indicated the intercalated structure of the azobenzene. It was confirmed that the initial position of the azobenzene did not significantly affect the energy-minimized structures, as long as the azobenzene was placed between the base pairs before calculation.

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Supporting Information Available: Tables listing T_m's of triplexes formed from X^mT_{13} and various duplex targets and MALDI-TOFMS results of modified oligonucleotides, and figures showing the hyperchromicity at 260 nm of the X^mT_{13}/a/t triplex, UV−vis spectra of *trans*-X^mT_{13}/a/t at 65 and 0 °C, and the melting curve for the triplex *trans*-X^mT_{13}/a/t monitored at 355 nm (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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