DNA Photocontrol

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Modulating PNA/DNA Hybridization by Light**

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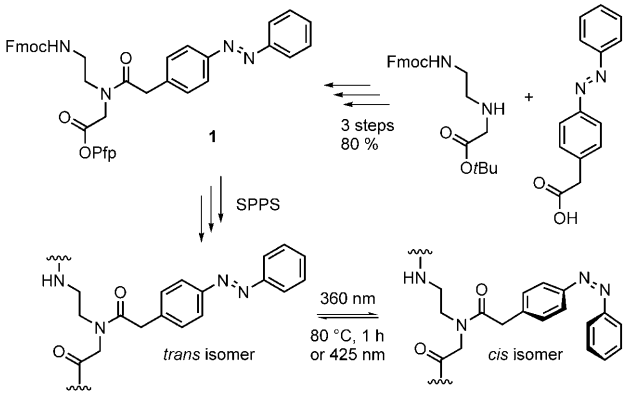
Photochromic compounds can be incorporated into oligonucleotides as light-activated switches for controlling structure and function with high spatial and temporal resolution.[1] In one approach, photolabile protecting groups have been successfully used to temporarily block binding of probe molecules to DNA or RNA targets until they are removed by irradiation.[1c] In another, reversible photoregulation of hybridization has been achieved by attaching moieties that undergo large light-induced geometrical changes to the nucleobases.[2,3] Here we show that the latter strategy is particularly effective with peptide nucleic acids (PNAs). Appending a single azobenzene photoswitch to a short PNA molecule provides excellent photocontrol over triplex binding and can be exploited for the photocontrol of transcription. Such compounds have considerable potential in biotechnology, diagnostics, nanotechnology, and medicine.

PNAs are synthetic nucleic acid analogs that present nucleobases on a repeating *N*-(2-aminoethyl)glycine polyamide backbone.[4] Though non-natural, these compounds hybridize to DNA and RNA through standard Watson–Crick and Hoogsteen base pairing.[5] Owing to their high affinity, sequence specificity, and physiological stability, PNAs are often superior to other nucleoside analogues for modulating diverse biochemical processes, ranging from transcription[6,7] and translation[8] to RNA splicing[9] and telomerase action.[10] In order to photoregulate such activities, we designed the Fmoc-protected, azobenenze-containing building block **1** (Scheme 1), which can be conveniently incorporated site-specifically into PNA oligomers.

Compound **1** is readily prepared in three steps from commercially available starting materials (see the Supporting Information for details). Its photochromic properties are similar to those of other azobenzene derivatives. Thus, at thermodynamic equilibrium greater than 90% exists in the *trans* configuration. Irradiation at 360 nm converts the *trans* into the *cis* isomer, providing up to 87% *cis*-azobenzene in the photostationary state, and this process can be reversed either thermally or upon irradiation at 425 nm. At the latter wavelength an 80:20 *trans*/*cis* mixture is generated.



Scheme 1. Azobenzene-modified building block **1** for incorporation into PNAs. By irradiation azobenzene can be switched between a planar *trans* and a bent *cis* isomer. Fmoc = fluorenemethylcarbamate, Pfp = pentafluorophenyl, SPPS = solid-phase peptide synthesis.

To assess the influence of the PNA photoswitch on nucleic acid hybridization, compound **1** was incorporated either at the N or C terminus of two representative PNA sequences, cattcatac and t_7, which form PNA/DNA duplexes and PNA_2/DNA triplexes, respectively, with complementary single-stranded oligonucleotides. As is common for PNAs, the oligomers were further modified with an *N*-acetyl group, a C-terminal carboxamide to remove a negative charge, and lysine residues to improve solubility (Table 1). Analogous compounds lacking the photoswitch were used as controls.

All peptide nucleic acids were synthesized by standard solid-phase methods[11] in good yield and purified by HPLC methods. For hybridization studies, the PNA derivatives were either irradiated at 360 nm or heated to 80 °C for 1 hour to generate photostationary or thermal equilibria that are enriched in the *cis* or the *trans*-azobenzene isomers, respectively. They were then added to sequence-complementary single-stranded DNA in neutral phosphate buffer, and complex formation was monitored spectroscopically as a function of temperature. All thermal melting profiles showed single sigmoidal transitions, allowing the determination of standard thermodynamic parameters from the concentration dependence of the melting temperatures (T_m).[12] Although *cis*-azobenzene reverts spontaneously and almost completely to the *trans* form (> 95%) if heated above 80 °C, it is sufficiently stable to provide reliable data as long as the T_m value is below 60 °C (the half-life of the *cis* isomer is 13.5 hours at 50 °C, see the Supporting Information).

The cattcatac PNA sequence forms antiparallel helical duplexes with DNA oligonucleotides like 5′-d(CATC**GTATGAATG**CTAC) containing a complementary pairing sequence (in bold). Appending the azobenzene photoswitch to either the N or C terminus of the PNA increases the

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Table 1: Parameters obtained from melting curves of the PNA/DNA hybrids.[a]

		Melting parameters		Stacking interaction		*cis–trans* discrimination	
	Azb	T_m [°C]	ΔG^{298} [kcal mol^{-1}]	ΔT_m [°C]	$\Delta\Delta G^{298}$ [kcal mol^{-1}]	ΔT_m [°C]	$\Delta\Delta G^{298}$ [kcal mol^{-1}]
PNA/DNA duplex				5′-d(CATC**GTATGAATG**CTAC)			
AcGly-Lys-(**cattcatac**)-Lys-GlyNH$_2$	–	40.3	−10.7 ± 0.1	–	–	–	–
AcGly-Lys-(**cattcatac**Azb)-Lys-GlyNH$_2$	*trans*	47.6	−12.1 ± 0.2	7.3	−1.4	2.5	−0.2
	cis	45.1	−11.9 ± 0.3	4.8	−1.2		
AcGly-Lys-(Azb**cattcatac**)-Lys-GlyNH$_2$	*trans*	46.8	−11.8 ± 0.2	6.5	−1.1	4.2	−0.7
	cis	42.6	−11.1 ± 0.2	2.3	−0.4		
PNA$_2$/DNA triplex				5′-d(CGTT**AAAAAAA**TTGC)			
Ac(**ttttttt**)-Lys-Lys-GlyNH$_2$	–	50.8	−22.2 ± 0.2	–	–	–	–
Ac(**tttttt**Azb)-Lys-Lys-GlyNH$_2$	*trans*	66.0	−29.7 ± 0.4	15.2	−7.5	13.4	−6.7
	cis	52.6	−23.0 ± 0.4	1.8	−0.8		
Ac(Azb**ttttttt**)-Lys-Lys-GlyNH$_2$	*trans*	59.5	−25.1 ± 0.6	8.7	−2.9	7.3	−3.5
	cis	52.2	−21.6 ± 0.5	1.4	+0.6		

[a] Melting temperatures (T_m) were determined in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0 using [CATC**GTATGAATG**CTAC] = 3.0 μM, [PNA] = 3.0 μM for duplex-forming PNAs, and [CGTT**AAAAAAA**TTGC] = 3.0 μM, [PNA] = 6.0 μM for triplex-forming PNAs; indices: *cis* = photostationary equilibrium (360 nm) with *cis*/*trans* 87:13; *trans* = thermal equilibrium (80 °C) *cis*/*trans* 5:95; standard errors for the melting temperatures (T_m) were estimated to be ±0.5 °C; the errors for the free energy changes ΔG^{298} correspond to the standard deviation from the mean average of at least four independent experiments; stacking interaction: $\Delta\Delta G^{298} = (\Delta G^{298}$ with modification minus ΔG^{298} without modification) and $\Delta T_m = (T_m$(modified) − T_m(reference)); *cis–trans* discrimination: $\Delta T_m = (T_m{}^{trans} - T_m{}^{cis})$; $\Delta\Delta G^{298} = (\Delta G^{298}$(trans) − ΔG^{298}(cis)); Gly = glycine; Lys = lysine; Ac = *N*-acetyl; NH_2 = carboxamide; incorporation of **1** is indicated by Azb.

stability of the resulting duplex by 0.4 to 1.4 kcal mol^{-1} (Table 1). These values are comparable to those provided by adding dangling benzene ($\Delta\Delta G = -0.7$ kcal mol^{-1}) and naphthalene ($\Delta\Delta G = -1.45$ kcal mol^{-1}) groups to duplex DNA.[13] The planar *trans*-azobenzene isomer is more stabilizing than the bent *cis* isomer by 0.2 to 0.7 kcal mol^{-1}, reflecting its ability to make better stacking interactions with the adjacent base pair. Although net stabilization is greater when the photoswitch is attached to the C terminus of the PNA, the *cis–trans* discrimination is superior at the N terminus. Overhanging nucleotides have negligible influence on the integrity of the PNA/DNA duplex as shown by the virtually unchanged ΔT_m values and stabilization energies observed with the shorter 5′-d(**GTATGAATG**) oligomer (see the Supporting Information).

Triplex formation is subject to more dramatic photocontrol. A t_7 PNA forms stable right-handed PNA$_2$/DNA triple helices with 5′-d(CGTT**AAAAAAA**TTGC) (Figure 1). Attaching *trans*-azobenzene to the PNA segment significantly stabilizes the triplex ($\Delta\Delta G = -2.9$ to -7.5 kcal mol^{-1}), whereas the *cis* isomer can stabilize or destabilize the structure depending on the site of modification ($\Delta\Delta G = -0.8$ to $+0.6$ kcal mol^{-1}; Table 1). As a consequence of these trends, the complex with a C-terminal *trans*-azobenzene is 6.7 kcal mol^{-1} more stable than that with the *cis* isomer. This translates into a 13.4 °C higher T_m value (Figure 1). *cis–trans* Discrimination is somewhat lower if the photoswitch is located at the N terminus of PNA, but still appreciable ($\Delta\Delta G = -3.5$ kcal mol^{-1}). These results can be contrasted with the photoregulation of triplex formation by DNA-based oligomers, which results primarily from steric destabilization of the complex by the *cis*-azobenzene isomer.[2c] The *trans*-azobenzene apparently magnifies the inherently greater stability of the PNA$_2$/DNA triplex relative to analogous DNA$_3$ structures,[15] allowing the use of considerably shorter

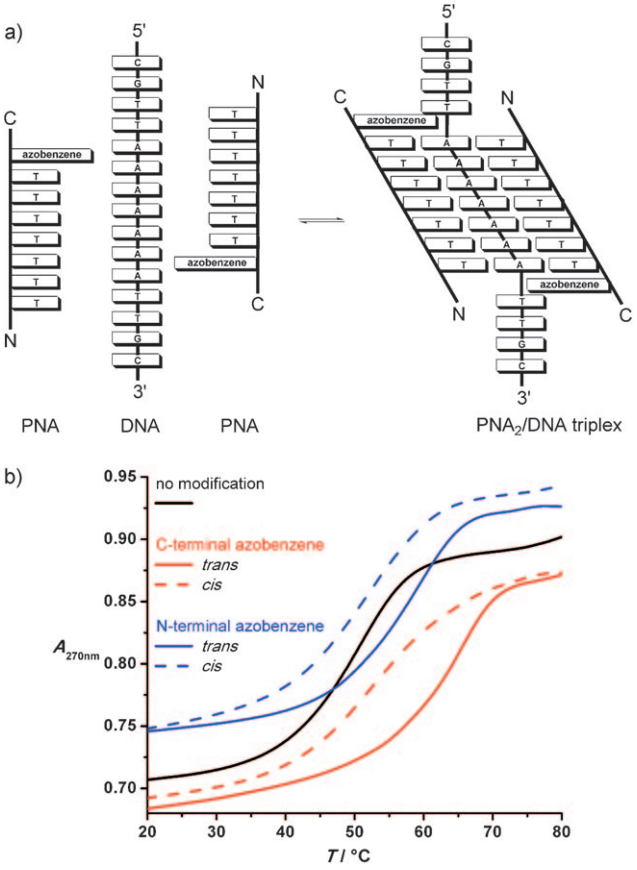

Figure 1. Photoswitchable PNA$_2$/DNA triplexes. a) Binding of an azobenzene-containing PNA to DNA to form a PNA$_2$/DNA triplex. b) Influence of the azobenzene photoisomers on UV melting curves of PNA$_2$/DNA triplexes; [DNA] = 3.0 μM, [PNA] = 6.0 μM in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0; absorbance 270 nm, heating curve (0.5 °C min^{-1}).

oligonucleotides and lower Mg^{2+} concentrations for recognition of target sequences. The latter features represent potentially significant advantages with respect to biological applications.

The efficacy of the triplex-forming photoswitchable PNA presumably derives from several factors. As in the duplex structures, the planar *trans* isomer is expected to stack better on the terminal base triplet than the sterically more demanding *cis* isomer, thereby preventing end fraying.[12a] Since a single base triplet contributes significantly more than a duplex base pair to complex stability ($\Delta G = -2.8$ kcal mol^{-1} [16] versus -1.4 kcal mol^{-1} [4a]), the net energetic gain is substantially greater. The presence of azobenzene units at both the 3′- and 5′-ends of the triplex further amplifies this effect. As a result, excellent *cis–trans* discrimination is achieved.

Triplex-forming PNA structures are potentially interesting as antigene agents since strand invasion of double-stranded DNA has been shown to arrest transcription in vivo.[6a] To investigate the feasibility of regulating this basic biological process by light, we examined the T7 RNA polymerase-catalyzed transcription of two genes, *efgp* (960 nt) and *torA* (414 nt), in the presence of a short ct_5c PNA probe with and without a C-terminal azobenzene moiety (Figure 2a). The *efgp* gene contains the complementary 5′-d(GA_5G) target sequence 300 nt downstream of the transcription start site. The *torA* gene, which lacks the recognition site, was used as a control for nonspecific binding. Each gene was expressed from a circular plasmid under the control of the T7 promotor and a T7 terminator. The effect of the probe on transcription was analyzed by separating the resulting RNA transcripts by agarose gel electrophoresis (Figure 2b and c).

As shown in Figure 2b, addition of the PNA probes (0 to 125 μM) inhibits transcription of the *efgp* gene in a concentration-dependent fashion. The probe concentration at which transcription is reduced by half (IC_{50}) depends on the presence and the configuration of the azobenzene moiety (Figure 2b) and follows the pairing energetics with a DNA oligonucleotide containing the target sequence, namely 5′-d(TCTT**GA_5G**TCAT). The *trans*-azobenzene probe binds most tightly ($T_m = 58$ °C) and is the most potent inhibitor ($IC_{50} \approx 35$ μM); the weaker binding *cis*-azobenzene probe ($T_m = 45$ °C) is more than two times less effective ($IC_{50} \approx 90$ μM), while the reference PNA lacking the photoswitch ($T_m = 40$ °C) is the poorest inhibitor ($IC_{50} > 125$ μM). Mutating the binding sequence on the *egfp* template [5′-d(GAAAAAG)→5′-d(GAACGCG)] significantly reduces inhibition (Figure 2c). Only at high PNA concentrations (≥ 100 μM) does nonspecific binding lead to a reduction in transcript yield. Transcription of the *torA* gene, which does not contain the target site, is even less sensitive to the presence of the probe. A scrambled PNA sequence (ct_5c-Azb→ct***g*t*a***tc-Azb) similarly fails to interfere with transcription of either gene below 100 μM (Figure S22 in the Supporting Information). Together, these results show that the PNA derivatives interact site-specifically with the target ds DNA template.

The ability of these short, triplex-forming PNA molecules to block gene expression is notable in light of the fact that analogous oligonucleotide-directed DNA-based triple helices fail to detectably inhibit transcription elongation.[17] Moreover, the sensitivity of inhibition to the configuration of the appended azobenzene establishes the feasibility of light-dependent transcriptional regulation. Nevertheless, the two-fold *cis–trans* discrimination observed in the transcription assay is substantially lower than might have been expected based on the thermodynamics of triplex binding alone, reflecting the difficulty of efficiently trapping single-stranded DNA in the transcription bubble of an actively transcribed gene. This complex process depends on the kinetics of strand invasion as well as on the stability of the resulting triplex structure.[16] To exploit light-mediated switching for practical applications, further optimization of the probe molecules will therefore be necessary. Based on work on RNA-binding PNAs,[8,9] it should be possible to increase the selectivity and affinity of the probes significantly simply by using longer sequences. Tighter binding PNAs would simultaneously minimize problems associated with nonspecific binding since less of the antigene PNA would be needed to achieve recognition of the target gene. Since formation of triplex invasion complexes is relatively slow and strongly concen-

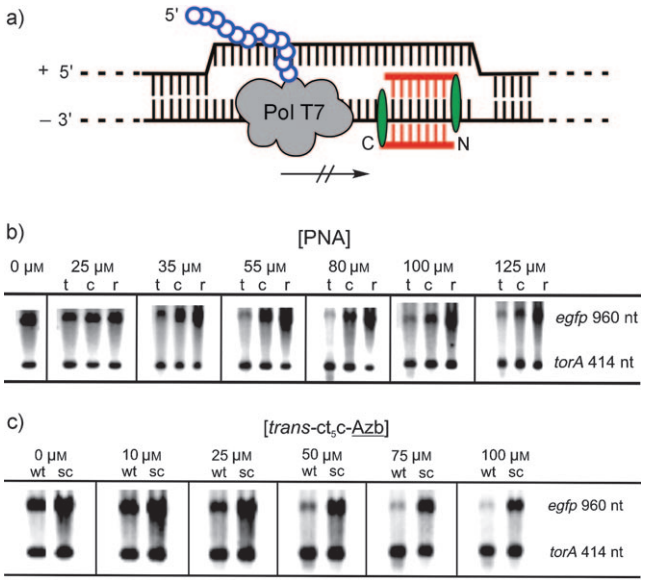

Figure 2. Inhibition of transcription by modified PNAs. a) PNA derivatives (PNA shown in red, azobenzene moiety in green) bind to their target sequence on the plasmid, 300 nt downstream of the T7 promotor, by strand invasion. The resulting PNA_2/DNA triplex blocks progression of the T7 RNA polymerase along the gene, causing premature transcription termination. b) Formation of full-length RNA transcripts from the *egfp* (960 nt) and *torA* (414 nt) genes was monitored by agarose gel electrophoresis on 2% agarose gels in 0.5×TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.3), 100 V. The PNA probe was added to the transcription mix at increasing concentrations (0 to 125 μM), leading to gradual disappearance of the *egfp* transcript. t = ct_5c PNA containing *trans*-azobenzene at its C terminus (thermal equilibrium: 1 h at 80 °C); c = PNA containing C-terminal *cis*-azobenzene (continuous irradiation at 360 nm); r = reference PNA lacking the photoswitch. Expression of the *torA* gene served as an internal control for nonspecific binding. c) The specificity of inhibition was investigated by comparing transcription from the wild-type (wt) *egfp* gene, an analogous template containing the scrambled (sc) binding sequence [5′-d(GAAAAAG)→5′-d(GAACGCG)], and the *torA* gene in the presence of the *trans*-ct_5c-Azb probe.

tration-dependent,[16] bisPNA[5,14a] or tail-clamp motifs[8b] in which triplex and duplex-forming domains are connected by means of a linker are attractive alternative probe formats. The sensitivity of the tail strand in tail-clamp PNA to structural modifications could be useful for enhancing photocontrol over the formation and disassembly of the triplex structures. Incorporation of azobenzene moieties at an interior site within the PNA sequence, rather than at the termini, or at multiple sites within the probe, would also be expected to amplify *cis–trans* discrimination as seen previously for DNA-based antigene sequences.[2a]

In conclusion, azobenzene-modified PNAs exhibit useful photoresponsive hybridization behavior. Their salient properties can be summarized as follows:

1) The *trans* form of the azobenzene PNA photoswitch stabilizes complexes more strongly than the *cis* form.
2) The magnitude of the *cis–trans* discrimination is site-dependent.
3) Discrimination is substantially stronger in the triplex than in the duplex binding mode.
4) *cis–trans* Discrimination in the triplex binding mode can be exploited to control transcription with light.

Because triplex-forming PNAs have been shown to exert strong inhibitory effects on translation,[8b] reverse transcription,[7] and replication,[14b] optimizing the properties of azobenzene-containing PNAs may provide useful photocontrol over these processes. Their inherent stability and cell permeability should make modified PNAs attractive alternatives to currently available photoswitchable DNA- and RNA-based systems for in vivo applications.

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