Photochromism

A Fast, Visible-Light-Sensitive Azobenzene for Bioorthogonal Ligation

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Abstract: Azobenzenes have been used as photoresponsive units for the control of numerous biological processes. Primary prerequisites for such applications are site-selective incorporation of photoswitchable units into biomolecules and the possibility of using non-destructive and deep-tissue-penetrating visible light for the photoisomerization. Here we report a push–pull azobenzene that readily undergoes a Staudinger–Bertozzi ligation with azide groups, that can be addressed with visible light (>440 nm) and exhibits the solvato- and acidochromism typical for push–pull systems. The thermal relaxation in aqueous environment proceeds on the low-millisecond timescale, thus enabling control over biological processes on similar timescales. The approach is demonstrated in the modification of a quartz surface and in the incorporation of an azobenzene unit into a functional peptide, the third zinc finger in the mammalian factor Sp1.

The azobenzene chromophore undergoes a cis/trans isomerization upon irradiation with UV light, which results in substantial changes in UV/Vis absorption, molecular geometry, and polarity. This phenomenon has placed the azobenzene photochromic units at the center of efforts towards photocontrol of biological processes, [1] such as nociception, [2] enzymatic activity, [3] membrane pore and channel function, [4] and cell adhesion. [5] Many of these applications rely on the site-selective introduction of the photoswitch into the structure of a biomolecule, such as a protein or DNA. Switching the molecules with less damaging and deeper penetrating visible light remains a major challenge for biomedical applications, and is being actively studied. [6]

Recently, we have reported [7] a new family of azobenzenes that incorporate in their structure a moiety capable of engaging in the Staudinger–Bertozzi ligation [8] (e.g., compound 4 in Figure 1) and hence enable introduction [8] of the photochromic unit into azide-containing proteins in a bioorthogonal fashion in aqueous conditions. [9] The Staudinger–Bertozzi protocol allows the reaction to be carried out without the use of additives that are normally involved in copper-catalyzed azide–alkyne cycloaddition, [10] and facilitates the formation of a rigid linker between the biomolecule and molecular photoswitch, distinguishing the approach from strain-promoted azide–alkyne cycloaddition. [11]

We focus our attention on azobenzenes that isomerize under the irradiation with visible light to circumvent the limitations presented by the application of UV light in a biological context, such as light scattering [12a] and cellular toxicity [12b–c]. Introduction of a strong push–pull substituent pair into the azobenzene structure, usually with dialkylamines as electron-donating substituents, has been demonstrated to give a considerable bathochromic shift of the π–π* absorption band. [13] Furthermore, such photoswitches typically undergo rapid thermal reversion (i.e., cis/trans isomerization). [14] This offers a key bene-

![Figure 1. a) Model Staudinger–Bertozzi ligation of compounds 3–5 with benzyl azide. b) 31P NMR spectra obtained from monitoring the reactions of compounds 3–5 with benzyl azide (2 equiv; for details, see the Supporting Information).](image-url)
fit for modulating fast biological processes\textsuperscript{[1a,15]} such as those related to vision\textsuperscript{[15c]} circumventing the need to use two distinct wavelengths for two-way switching and allowing for rapid and complete resetting of the trans state in the dark.

Herein, we demonstrate a phosphine-functionalized azobenzene for use in Staudinger–Bertozzi ligation (compound 3, Scheme 1), which upon reaction with an azide group forms a visible-light-responsive (\(\lambda > 400\) nm) switchable chromophore, which undergoes rapid (several milliseconds) thermal reversion from the cis isomer to the stable trans-isomer in an aqueous medium.

Compound 3 is based on the structure reported by our group recently (i.e., compound 4, Figure 1),\textsuperscript{[17]} in which the aromatic moiety that partakes in Staudinger–Bertozzi reaction is combined with an azobenzene unit. The p-dialkylamino group was introduced to create a strong push–pull type of photochromic unit, with the aim of shifting the absorption maximum bathochromically and enabling fast thermal cis\textendash;trans isomerization.

Compound 3 was synthesized from precursor 1 in a two-step procedure (Scheme 1).\textsuperscript{[16]} Diazotisation of 1, followed by reaction with \(N,N\)-diethylaniline gave compound 2, which was converted to 3 by palladium-catalyzed cross-coupling with diphenylphosphine.

The efficiency of 3 in the ligation to azides was evaluated and compared with systems reported elsewhere (Figure 1a)\textsuperscript{[17,19]} \(\text{\textsuperscript{31}P}\) NMR spectroscopy\textsuperscript{[17]} proved to be convenient and compared with systems reported elsewhere (Figure 1a).\textsuperscript{[7,17]} The numbers in parentheses correspond to the \(\lambda_{\text{max}}\) of the UV/Vis absorption spectra of 6 (3.0 \times 10^{-4} \text{ M}) obtained in various solvents at room temperature (buffer: 5 mM TRIS\textcdot\text{SO}_4, pH 7.2); b) UV/Vis absorption spectra of 6 (1.0 \times 10^{-3} \text{ M}) obtained in 10 vol\% MeCN in water with increasing pH (for details, see the Supporting Information).

The UV/Vis absorption spectra of compound 6 were recorded in various solvents (Figure 2a), to verify if the expected solvatochromic effects reported for other push–pull azobenzenes were manifest.\textsuperscript{[13,18]}

The position of the absorption maximum of 6 was found to correlate with solvent polarity (Figure 2a). The absorption spectrum of 6 was most blueshifted in dioxane; the least polar of the solvents used (\(\varepsilon = 2.3\)). Compound 6 showed a gradual bathochromic shift over the series of protic solvents as the polarity increased, in the order of \(\text{iso-propanol} (\varepsilon = 18)\), ethanol (\(\varepsilon = 24.5\)), and methanol (\(\varepsilon = 33\)). The absorption spectrum in acetone (\(\varepsilon = 37.5\)) was similar to that in methanol. In the aqueous buffer with 10 vol\% of acetoneitrile, the spectrum was most bathochromically shifted. These observations are in agreement with the proposed increase of the contribution of the zwitterionic resonance structure \(\text{IIb} (\text{Scheme 3})\) in solvents of higher polarity.\textsuperscript{[13,18,c]}

The substantial bathochromic shift of the absorption maximum, observed for the products of the coupling of compound 3 with azides, enables the use of visible light to induce isomerization, which allows deeper-tissue penetration and much less risk of toxicity.

Titrations of push–pull azobenzenes with acid, resulted in the formation of several possible species\textsuperscript{[18c,19]} (Figure 2b and Scheme 3). The mono-protonated species exists as an equilibrium mixture of two tautomers: ammonium ion II and azonium ion III (Scheme 3), with distinctly different spectral properties.\textsuperscript{[19]} The formation of the ammonium ion II resulted in the loss of electron-donating properties and
hence the push–pull character of the azobenzene, manifested in a hypsochromic shift of the absorption spectrum. Protonation of the diazo group, giving the azonium ion III, leads to reinforcement of the push–pull system, through resonance stabilization, and a bathochromic shift of the absorption spectrum occurs.

The pH dependence of the UV/Vis absorption spectrum of 6 showed a gradual bathochromic shift of the spectrum, indicative of the formation of an azonium ion III (Figure 2b). This observation is consistent with other reported push–pull azobenzenes. In strongly acidic media, the spectral changes suggest double protonation to form structure IV (Figure 2b, pH 0.7).

The effect of addition of Lewis-acidic metal cations, often present in the aqueous solutions used in biological studies, on the UV/Vis absorption spectrum of compound 6 was examined (Figure 3). The titration curve obtained with CaCl₂ was indicative of complex formation (Figure 3). With Na⁺, analogous changes in absorption maximum were not observed over the concentration range studied (Figure 3). The preferential binding of divalent metal cations could be explained by the proximity of two Lewis-basic residues in 6, that is, the oxygen atoms of the amide and the phosphine oxide moieties that can act as ligands for Ca²⁺. Complexation of metal ions would result in the enhancement of the push–pull character of the system, through increased contribution of Ib/IIIb-type resonance structure, leading to a bathochromic shift in the spectrum (Scheme 3). Alternatively, the shift might be explained by the coordination of Lewis acidic cations to the diazo group.

The spectral properties of a model ligation product 6 depend on the polarity of the solvent, the pH, and the presence of Lewis acids, which has implications for the studies on the speed of thermal cis/trans isomerization of 6. Specifically, the bathochromic shift of absorption maximum in more polar solvents (Figure 2a), at lower pH (Figure 2b) and at higher concentrations of divalent cations (Figure 3) suggests a possibility of fast cis/trans thermal isomerization (see below) due to the loss of N=N double bond character in resonance structures Ib and IIIb (Scheme 3).

With this in mind, the photo- and thermal switching of compound 6 was studied as a monolayer of a covalently bound azobenzene switch on a quartz-cover slip. A piranha-cleaned quartz-cover slip (A, Scheme 4) was decorated with azide groups by reaction with azido–silane 7 to give surface B (Scheme 4). Subsequent modification of the surface by Staudinger–Bertozzi ligation with compound 3 gave surface C (Scheme 4). The modification was confirmed by an appearance of a UV/Vis absorption in the range λ = 450–550 nm (see the Supporting Information). Furthermore, a lower contact angle measured with HCl (1 n aq.) was observed compared...
Figure 4. Thermal cis/trans isomerization of compound 6. a) $3 \times 10^{-3} \text{ M}$ in methanol at 183 K, monitored by the absorbance at $\lambda = 450 \text{ nm}$. The cis form was generated by irradiation with a Hg lamp with 400 nm long pass filter; b) $1.30 \times 10^{-3} \text{ M}$ in methanol at 293 K, monitored at $\lambda = 430 \text{ nm}$ following excitation at 532 nm (6 ns full width at half maximum (FWHM), 10 Hz, 3 mJ); c) $7.0 \times 10^{-3} \text{ M}$ in 10% MeCN/5 mM TRIS·SO$_4$, pH 7.2 at 293 K, recorded at $\lambda = 460 \text{ nm}$ upon 532 nm (6 ns FWHM, 10 Hz, 3 mJ). Points correspond to measured data; lines represent the fitting with single exponential decay.

with water, which is consistent with the presence of basic amino groups that increase the hydrophilicity of the surface upon protonation (Scheme 4). Unfortunately, the reversible photoisomerization on the surface could not be confirmed, because no significant changes in the UV/Vis spectra and contact angle upon visible-light irradiation were observed.

The reversible photoisomerization of 6 was studied at 183 K in methanol (Figure 4a). Irradiation resulted in a decrease in the absorbance at $\lambda = 450 \text{ nm}$, indicative of the photoinduced cis/trans irradiation (see the Supporting Information). The thermal cis/trans reisomerization was observed to have a lifetime ($1/k$) of $r = 17 \text{ s}$ at this temperature (Figure 4a). The lifetime for the thermal process in methanol at room temperature was measured by laser-flash photolysis (Figure 4b). The lifetime for the thermal process was determined to be $40 \pm 3 \text{ ms}$. Due to the fast relaxation, the content of the cis-isomer in the photoradiated sample could not be determined.

The fast thermal isomerization observed in solution suggests strongly that the absence of observable photochemistry in the case of the monolayer formed on quartz was due to limitations in the intensity of irradiation that could be employed.

Envisioning the application of the above-described, fast molecular photoswitches in biological (aqueous) setting, we studied the stability of the cis isomer at room temperature in TRIS·SO$_4$ buffer (TRIS = tris(hydroxymethyl)aminomethane) with acetonitrile as a co-solvent (Figure 4c). In this highly polar environment, the thermal cis/trans isomerization was much faster than in methanol, with a sub-millisecond lifetime (Figure 4c). This kinetics allows high temporal selectivity in the photoccontrol of fast biological processes, for example, those connected to vision.

Furthermore, the possibility of introducing the azobenzene tag 3 to an azide-decorated, functional peptide in aqueous conditions, was studied. As a model, the third zinc finger in the mammalian factor Sp1 (Sp1-f3) was chosen. It belongs to the family of C$_2$H$_2$-type zinc fingers, which are small DNA-binding motifs that mediate DNA–protein interactions within cells, playing an important role in regulation of DNA expression. It has been already shown that incorporation of azobenzene moiety into the N-terminus of Sp1-f3 allows a partial photocontrol over the binding of zinc finger to DNA.

By using solid-phase peptide synthesis (SPPS), a modified variant of Sp1-f3 was prepared, in which l-azidohomoalanine 8 was introduced in position 27 ([Aha$^{27}$]-Sp1-f3, Scheme 5 and Figure 5a). The modification point was chosen close the $\alpha$-helix region of the zinc finger, which is directly involved in the interactions with DNA.

The reaction of [Aha$^{27}$]-Sp1-f3 with compound 3 was followed by HPLC and MS analysis (Figure 5a–c). After two days (Figure 5b), the product was formed, and after purification (Figure 5c) it was subjected to MS analysis and was confirmed to be the expected Peptide 1, with the azobenzene tag introduced via Staudinger–Bertozzi ligation (Scheme 5). The incorporation of visible-light switchable azobenzene into the biologically functional peptide (Figure 5d). We are currently investigating the possibility of using Peptide 1 for the light-controlled binding to DNA.

In summary, a visible-light-sensitive, push–pull azobenzene photoswitch 3, which can be conveniently introduced into the mammalian factor Sp1 (Sp1-f3) was chosen. It belongs to the family of C$_2$H$_2$-type zinc fingers, which are small DNA-binding motifs that mediate DNA–protein interactions within cells, playing an important role in regulation of DNA expression. It has been already shown that incorporation of azobenzene moiety into the N-terminus of Sp1-f3 allows a partial photocontrol over the binding of zinc finger to DNA.

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Scheme 5. Introduction of an azobenzene tag to azidohomoalanine-modified Sp1-f3 zinc finger (structure adapted from pdb file, entry 1SP1[1]).
azide-containing targets by using Staudinger–Bertozzi ligation, without the use of additives or catalysts, was presented herein. The model product 6, formed upon ligation, has an absorption maximum in the visible range ($\lambda > 440$ nm) and shows the expected solvato- and acido-chromism. Specifically, in media with higher polarity and acidity, a bathochromic shift was observed. The position of the absorption band can also be influenced by the addition of divalent metal cations.

At lower temperature, the light-induced, reversible changes in UV/Vis spectra that indicate the cis/trans isomerization, were observed. Using laser-flash photolysis, this isomerization was also confirmed at room temperature in methanol and aqueous solutions. In the latter case, fast thermal relaxation of the cis-isomer was observed, with a lifetime of the order of 0.5 ms.

To confirm that compound 3 can be used for the modification of biomolecules, we present its application for site-selective incorporation of the photochromic residue into the structure of azide-modified zinc finger protein. We envision the application of the presented Staudinger–Bertozzi azobenzene photoswitches in the photoscontrol of fast biological processes, due to the reversible, visible-light-induced switching process and fast thermal relaxation in aqueous environment.

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