Photoisomerism

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Photoswitching of *ortho*-Substituted Azonium Ions by Red Light in Whole Blood**

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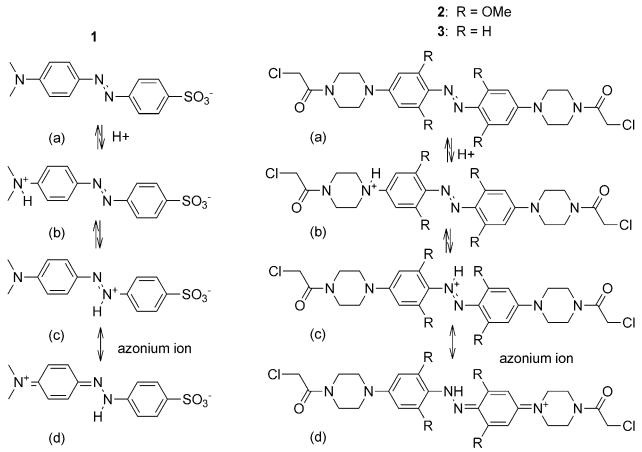
Photocontrol using red light is highly desirable for biological applications, as red wavelengths are the only part of the visible spectrum that can effectively penetrate tissue.[1] Efforts to develop optogenetic and optochemical genetic methods that are red-shifted range from exploring natural biodiversity in the search for red-shifted opsins[2] to the conjugation of chemical photoswitches to upconverting nanoparticles.[3] We recently reported that azobenzenes with bulky polar substituents in all four positions *ortho* to the azo group could undergo red-light-driven photoisomerization.[4] However, the compounds require intense red light or long irradiation times to reach the photostationary state because the absorption coefficients for wavelengths of more than 600 nm are very small.[4]

Azonium ions formed by amino-substituted azobenzenes (Scheme 1) are well-known species that have strong absorbance in the red region of the spectrum.[5] Two features, however, make typical azonium ions difficult to use as photoswitches in a biological context. First, most azonium ions, such as protonated methyl orange (**1**), have pKa values in the range of 1.5–3.5[5a–c,6] so that the azonium species is hardly present at the neutral pH normally encountered in vivo. Second, the *cis*-to-*trans* thermal isomerization rate of azonium ions is fast, with *cis* half-lives in the μs range, so that production of a significant concentration of the *cis* isomer is difficult without very bright light sources.[7] The rapid thermal isomerization of the *cis* azonium species is attributed to the decreased double-bond character of the N–N bond owing to the contribution of resonance structure **1**(d).[8]



Scheme 1. Neutral and protonated forms of methyl orange (**1**) and the *ortho*-substituted (**2**) and unsubstituted (**3**) aminoazobenzenes studied herein. Diprotonated species can also form (see Ref. [5a]).

We report herein that introduction of methoxy substituents to all four positions *ortho* to the azo group in an aminoazobenzene derivative has a remarkable effect on the photochemistry in that it enables photoswitching of the related azonium ion at neutral pH with red light. We synthesized the tetra-*ortho*-methoxy substituted aminoazobenzene derivative **2**, a structure that permits two-point attached to a thiol containing target biomolecule. Synthesis of **2** was initiated from 1-bromo-3,5-dimethoxybenzene, which was transformed into 2,2′,6,6′-tetramethoxy-4,4′-dibromoazobenzene, as described previously.[4] Palladium-catalyzed twofold amination at the 4 and 4′ positions with *tert*-butylpiperazine-1-carboxylate led to di-*tert*-butyl-4,4′-(diazene-1,2-diylbis(3,5-dimethoxy-4,1-phenylene))bis-piperazine-1-carboxylate (see the Supporting Information). Boc deprotection of amines followed by chloroacetylation using chloroacetyl chloride resulted in (**2**), which was then reacted with a test peptide AB15 (Ac-WGCAEAAAREAAAREAACRQ-NH_2) having Cys residues at i and $i+15$ positions.[9] Attachment to a peptide ensures water solubility and prevents self-association of the dye as well as mimicking the target environment for the photoswitch. Azobenzene derivatives attached as cross-linkers to peptides and proteins have been used to drive conformational and functional changes in a variety of targets.[1a,10]

Figure 1 shows UV/Vis spectra in the range pH 5–9 of **2** and **3** (the non-methoxy substituted counterpart[9]) in their *trans* states after attachment to AB15. For the tetra-*ortho*-methoxy-substituted compound **2**, the azonium ion ($\lambda_{max}=$ 560 nm) is produced with an apparent pK_a of 7.2. The corresponding non-*ortho*-substituted species **3** does not become protonated in this pH range (Figure 1b). The azonium ion of **3** is only seen below pH 3 (Supporting Information, Figure S1).

Irradiation of the azonium peak of **2** cross-linked to AB15 at pH 7.5 with red light (635 nm, 80 mW cm^{-2}) produces a marked photochromism (Figure 2a). The strong absorbance of the azonium species (ca. 20000 L mol^{-1} cm^{-1} at 600 nm, pH 7.0; see the Supporting Information) results in rapid (ca. 1 s) production of the photostationary state (Figure 2b).

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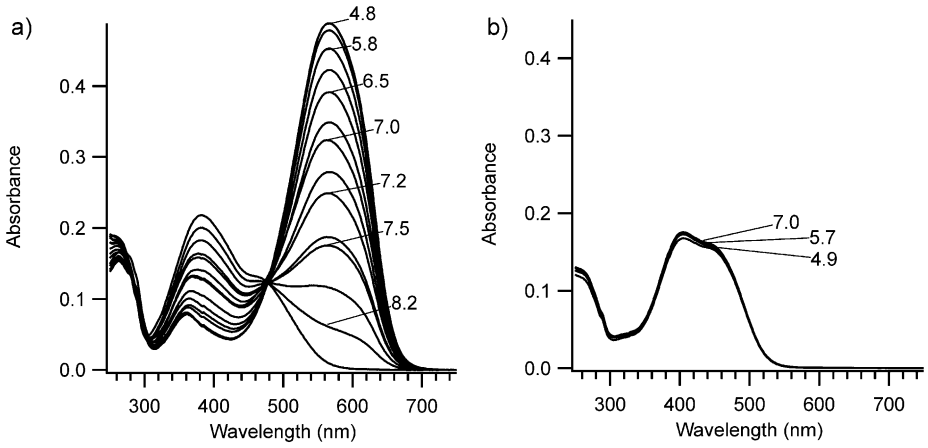

Figure 1. UV/Vis spectra of the *trans* isomers of a) **2** and b) **3** cross-linked to the peptide AB15 in aqueous buffer at the indicated pH values (22 °C).

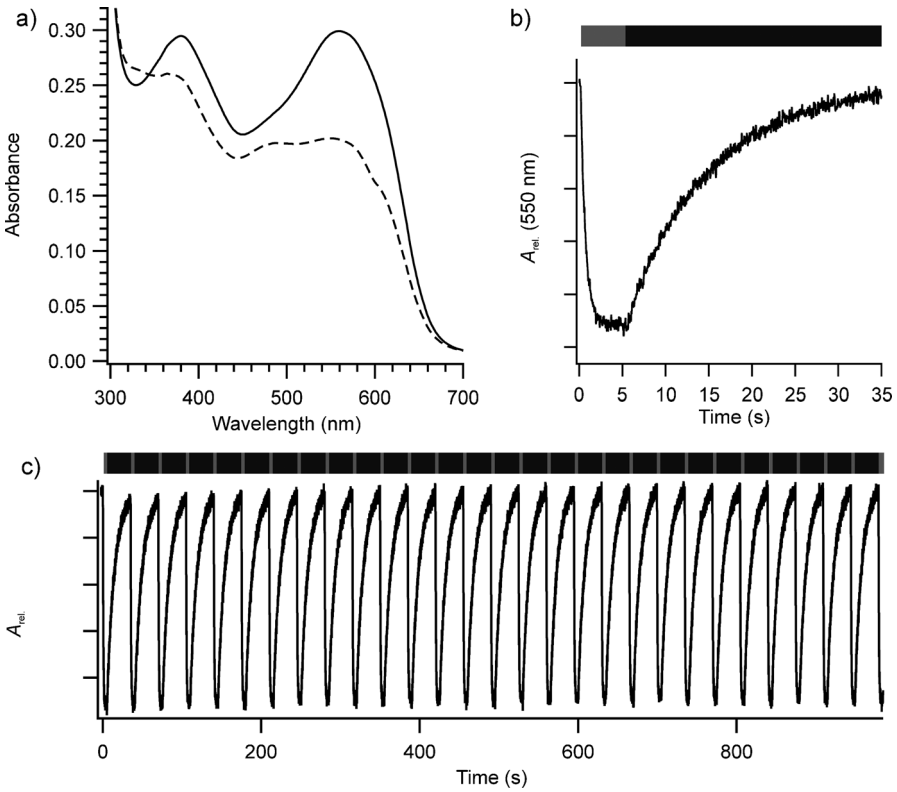

Figure 2. a) UV/Vis spectra of **2** cross-linked to the peptide AB15: (——) dark-adapted, (-----) with red-light irradiation (pH 7.5, 22 °C). b) Photoisomerization under red light (indicated by the gray bar) followed by thermal recovery in the dark (black bar). c) Multiple photoswitching cycles show no evidence of photobleaching (pH 7.5, 22 °C).

After removing the red-light irradiation, the dark-state spectrum recovers thermally in a monoexponential manner with a half-life of about 10 s at pH 7.5 (Figure 2b). This process can be repeated over many cycles with no apparent photobleaching (Figure 2c).

The thermal isomerization of methyl orange (**1**) has been studied in detail using laser flash photolysis techniques by Barra, Sanchez, and de Rossi.[7] These authors observed half-lives for thermal recovery by the azonium ion on the order of 2–3 μs. Thus, under comparable conditions, the thermal recovery rate of **2** is about 10^6 times slower. The consequence of this dramatically slowed thermal isomerization rate is that substantial populations of the thermally less-stable *cis* species can be produced by red LED illumination under physiological conditions.

In an effort to understand the remarkable behavior of **2**, we carried out DFT (B3LYP, 6-311++G(d,p)) calculations to find minimum energy structures (see the Supporting Information). We used piperidino analogues of **2** to simplify the calculations. As shown in Figure 3, the neutral species **2a** is non-planar (Figure 3a) with substantial twisting of the rings, as was observed in X-ray crystal structures of other neutral tetra-*ortho*-methoxy species reported earlier.[4] Calculations predict the azonium species **2**-(c/d), however, is planar (Figure 3b). Planarity facilitates resonance stabilization and H-bonding interactions as diagrammed in Figure 3c. The presence of an H-bond as shown in Figure 3b was confirmed by an atoms-in-molecules analysis[11](see the Supporting Information). Stabilization of azonium species by a single *ortho* substituent capable of H-bonding has been reported previously.[12] In **2**, however, these effects apparently raise the pK_a by about 4–5 pH units (compared to **3**), so that the azonium species is the dominant species at physiological pH values.

The red-light-induced photochromism observed (Figure 2) implies the *trans* azonium species can absorb red light and isomerize to produce a *cis* species that has a lower absorbance coefficient. Figure 4 shows calculated minimum energy structures for the protonated and neutral *cis* species (Figure 4a,b). Unlike the *trans* azonium species, the proton on the azo moiety of the *cis* azonium ion is too far from the *ortho* methoxy substituents to form an effective H-bond, suggesting the pKa of the *cis* azonium species is lower than the *trans*. DFT calculations also indicate the proton affinity of the *cis* isomer is lower than the *trans* (see the Supporting Information). Thus, photoisomerization to the *cis* azonium species may be accompanied by conversion to the neutral *cis* state. Time-dependent DFT calculations predict that both neutral and protonated *cis*

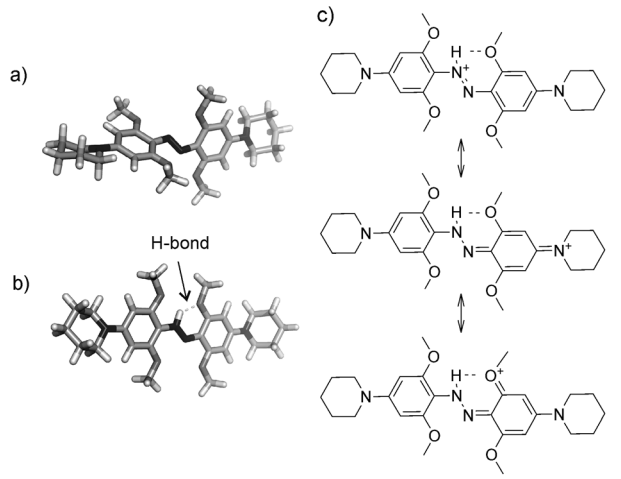

Figure 3. Calculated minimum-energy structures of *trans* neutral (a) and protonated (b) forms of a model compound representative of **2**. c) Resonance representations showing effects that stabilize the azonium ion.

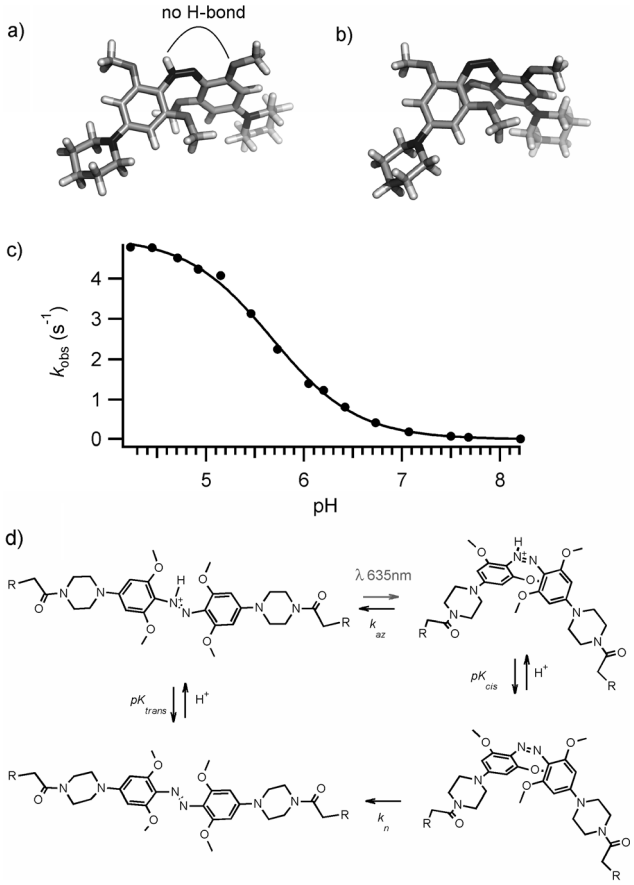

Figure 4. *Cis*-protonated (a) and neutral (b) forms of a model compound representative of **2**. c) Observed rate constant for thermal relaxation as a function of pH. Limiting half-lives are about 1 min for the neutral species and about 100 ms for the *cis* azonium ion. d) Kinetic model showing isomerization and protonation of **2** (R represents the linkage to the peptide). The data in (c) are fit to the following equation:
$k_{obs} = k_n\left(\frac{10^{-pK_{cis}}}{(10^{-pH})+(10^{-pK_{cis}})}\right) + k_{az}\left(\frac{10^{-pH}}{(10^{-pH})+(10^{-pK_{az}})}\right)$.

species have lower absorbance coefficients in the red region than the *trans* azonium ion (see the Supporting Information).

A neutral *cis* isomer would be expected to undergo thermal relaxation to the *trans* state much more slowly than the *cis* azonium species.[7,8] We measured the rate of thermal recovery as a function of pH (Figure 4c) and found a sigmoidal dependence to the observed rate constant. This behavior is consistent with the simple kinetic model shown in Figure 4d in which protonation/deprotonation rates are fast compared to rates of thermal relaxation and the neutral *cis* species undergoes relaxation (k_n) about 1000 times more slowly than the *cis* azonium ion (k_{az}). From these data, the *cis* azonium ion is estimated to have a pK_a of 5.7, which is lower by 1.5 pH units than the *trans* azonium ion. The million-fold decrease in the rate of thermal relaxation of **2** compared to typical azonium ions therefore appears to be due to two factors: 1) an intrinsic effect of the tetra-*ortho* substitution pattern on the thermal barrier; and 2) deprotonation accompanying conversion into the *cis* isomer at physiological pH values.

Red light has significantly higher penetration through biological tissue than other parts of the visible spectrum, which is primarily because wavelengths of more than about 600 nm can avoid absorbance by hemoglobin.[13] To test directly the possibility of using **2** as a photoswitch for controlling conformational changes and ultimately bioactivity in vivo, we mixed **2**-cross-linked peptide directly with whole blood. The azonium absorption band extends beyond the hemoglobin absorbance (Figure 5a). Irradiation of the

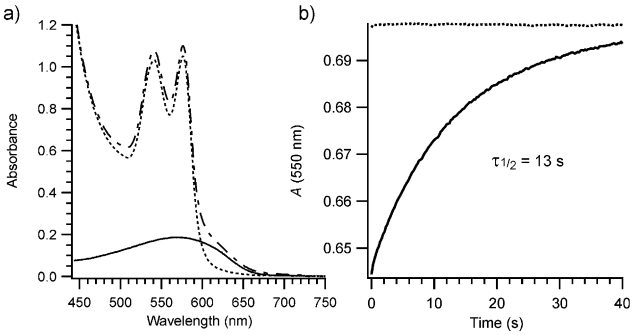

Figure 5. a) UV/Vis spectra of whole blood (••••), only AB15 cross-linked with **2** (——), and AB15 cross-linked with **2** (1 mM; –•–•) in whole blood. Each sample was diluted 1000-fold in Tris-buffered saline, pH 7.4 before scanning (b) Thermal relaxation after red-light irradiation (80 mWcm^{-2}, 635 nm) of blood only (••••) and undiluted blood containing AB15 (——) cross-linked with **2** (after incubation at room temperature for 12 h).

sample at 635 nm produced photoswitching that was stable for at least 12 h (Figure 5b).

The tetra-*ortho*-methoxy substitution pattern could be used to develop a class of azo compounds for which isomerization can be triggered by long-wavelength light. Azonium species are known with absorbance maxima of more than 660 nm[5b,d] and with further tuning of the pK_a difference between *cis* and *trans* isomers, azo compounds might be developed for manipulating biomolecules in vivo with near-

infrared light. As might be anticipated based on previous work,[4] there is some sensitivity to reduction by high concentrations of glutathione, however (the compound is reduced by 10 mM GSH with a half-life of about 1.4 h; see the Supporting Information), so that use may be restricted to extracellular and/or oxidizing environments in vivo. For example, blood-borne peptide hormones and growth factors could be coupled to an azonium-based photoswitch and manipulated non-invasively with a high degree of spatiotemporal control.

Experimental Section

The peptide AB15 was prepared using standard Fmoc-based solid-phase synthetic methods. Intramolecular cross-linking of cysteine residues on AB15 with **2** (freshly prepared, see details in the Supporting Information) was performed in 50% DMSO as follows: A solution of 0.5 mM peptide (freshly purified) and 2 mM cross-linker **2** in 50 mM Tris buffer at pH 8 was stirred at 40 °C under a nitrogen atmosphere for 16–20 h. The completion of the reaction was judged by MALDI mass spectra. The reaction was dried under high vacuum, and the cross-linked peptide was purified by reverse-phase HPLC on a semipreparative RX-C8 column (Zorbax, 9.4 mm ID × 255 mm) with a linear gradient of 10–65% acetonitrile/water (containing 0.1% trifluoroacetic acid) over a period of 25 min. Cross-linked AB15 was eluted at 47% acetonitrile. ESI-MS: *m/z* calcd for $C_{114}H_{173}N_{38}O_{34}S_2$: 2683.9 [$M^+$], observed: 2682.4.

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